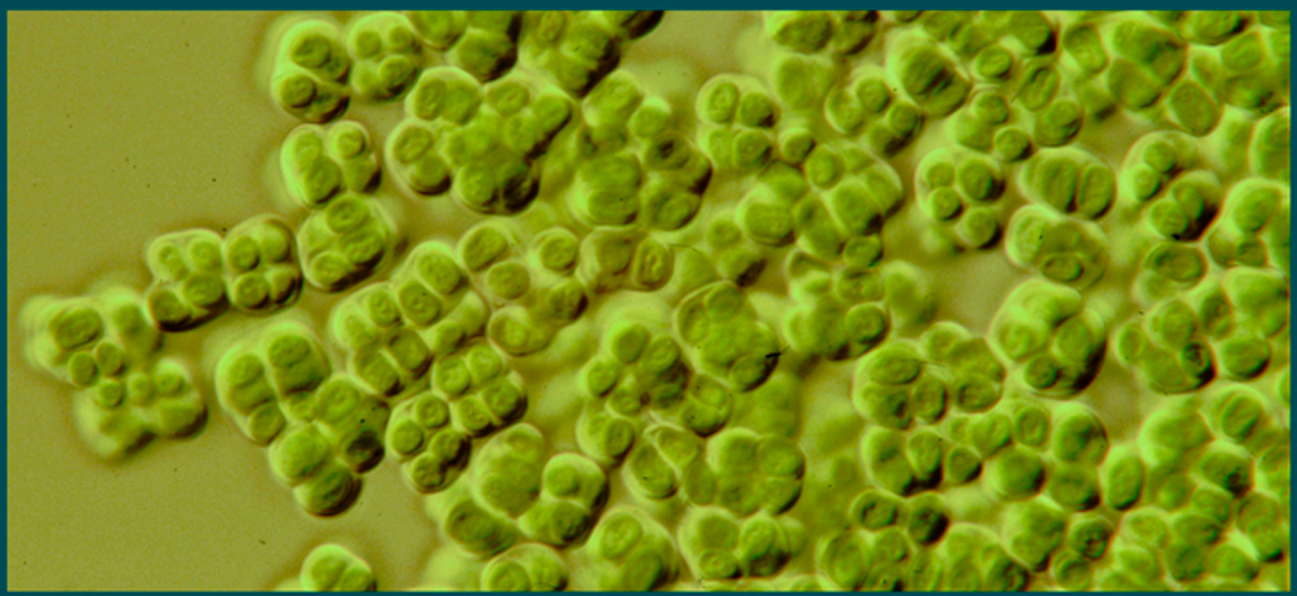
CYANOBACTERIA

an economic perspective

Edited by

Naveen K. Sharma, Ashwani K. Rai and Lucas J. Stal



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Cyanobacteria

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An Economic Perspective

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Preface

Human society faces enormous problems in the near future in order to cover the increasing demands of energy, food, and health care. The current ways these demands are covered by society are not sustainable and result in unacceptable changes in our environment, such as global warming due to increasing emissions of the greenhouse gases carbon dioxide, methane, and nitrogen oxides. The increasing emissions of carbon dioxide cause the acidification of the ocean with difficult-to-predict effects. The extensive and increasing use of freshwater and arable land for agriculture and for the production of biofuels compete with food production. The over-use of antibiotics, not only to defeat human illness and infections, but also to increase and economize animal production, has already lead to multiple resistant pathogens and therefore there is an urgent need to discover alternative medicines. These are, in a nutshell, a few of the challenges that human society is currently facing.

The Earth formed more than 4.5 billion years ago. The origin of life on Earth was probably around 4 billion years ago but the rock record goes back only 3.8 billion years and the organic matter in these rocks hints at carbon dioxide fixation. The oldest microfossils are found in lithified microbial mats – so-called stromatolites – dating back almost 3.5 billion years. These might have been cyanobacteria. Modern microbial mats and stromatolites are built by cyanobacteria and are analogues of those in the fossil record. Multicellular organisms such as the plants and animals only developed 0.6 billion years ago. Hence, life was microbial for at least 3.2 billion years, during which time it evolved a stunning genetic diversity. All biogeochemical cycles are run by microorganisms. The number of different types of microorganism and their genetic diversity is unknown but is estimated to be many tens or hundreds of millions, harboring a plethora of metabolic pathways with the capacity to produce bioactive compounds, as well as other possible uses that are awaiting to be discovered and used by human society.

Cyanobacteria are oxygenic phototrophic bacteria. They use water as the electron donor, splitting it into oxygen and hydrogen. The latter is used to fix carbon dioxide into organic matter using sunlight as the source of energy. Cyanobacteria were responsible for the oxygenation of the Earth's atmosphere 2.5 billion years ago, and led to the formation of eukaryotic algal and plant cells through an endosymbiotic event with a non-phototrophic host. The endosymbiotic cyanobacterium evolved into the chloroplast of algal and plant cells. It is estimated that cyanobacteria have produced half of global oxygen and plants and algae the other half, but as chloroplasts can be considered to be endosymbiotic cyanobacteria, essentially all oxygen that is produced on Earth is cyanobacterial.

Due to their long history, cyanobacteria have evolved a large morphological and genetic diversity and are known for the production of wide range of bioactive compounds and multiple biotechnological applications such as the production of biofuels (production of ethanol, butanol, or lipids) or food, food additives, and single-cell protein. Cyanobacteria can be grown in mass cultures and because they use sunlight, mass cultivation may be economic. Moreover, because cyanobacteria fix carbon dioxide, the biofuels they produce are carbon-dioxide neutral and sustainable. Cyanobacteria grow in virtually any illuminated environment. Hence there are many species that grow in seawater or at least are salt-water tolerant, eliminating

the use of precious freshwater supplies. Many cyanobacteria also grow under extreme conditions so that mass cultivation can be undertaken in areas that are not suitable for food production and hence does not compete with it. Also, the possibility of growing cyanobacteria under extreme conditions presents an important possibility for mass cultivation because it prevents infections and allows stable long-term cultivation.

During recent decades, much research has been carried out into the biotechnological applications of cyanobacteria. With this book we wanted to bring together this knowledge and present cyanobacteria from an economic perspective. We are grateful to the many contributors to this book who provided up-to-date overviews of the biotechnological potential of cyanobacteria and the problems with and feasible opportunities for economic industrial-scale cultivation of these organisms. The contributions in this book also review cyanobacteria from the taxonomic and ecological points of view. This information is crucial for strain selection, design of photobioreactors and planning of economic industrial-scale cultivation. The book also reviews the plethora of biotechnological applications of cyanobacteria, varying from pharmaceuticals, food, food additives, to biofuels and others. In addition, it discusses the possibility of designing cyanobacteria as cell factories by enhancing their metabolism through changes to their genetic content and regulation. We are convinced that this book will be an important resource for anyone who is interested in cyanobacteria and their biotechnological potential, and we express the hope that this book will stimulate and help scientists and biotech engineers to move this field into new and improved applications.

April, 2013

Naveen K. Sharma

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BIOGRAPHY

Prof. Ashwani K. Rai has more than 35 years of teaching and research experience at the Department of Botany, Banaras Hindu University. He has published more than 80 original research papers and reviews, authored/edited five books and supervised 15 doctoral theses. His area of research includes cyanotoxins, nitrogen metabolism, carbon fixation and salt tolerance in cyanobacteria. Professor Rai has been the recipient of several prestigious fellowships and awards including a Matsumae International Foundation Fellowship, Japan (1982); an Alexander von Humboldt Fellowship, Germany (1983); the National Biotechnology Overseas Associateship Award (DBT, New Delhi, 1986); an Indo-JSPS Exchange Fellowship

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BIOGRAPHY

Prof. Lucas J. Stal studied biology at the University of Groningen, The Netherlands where he obtained his Master's degree in 1978 specializing in Microbial Ecology and Molecular Genetics. Then he moved to Germany where he became assistant professor in Geomicrobiology at the University of Oldenburg. He obtained his PhD in 1985 from the University of Groningen for a study on nitrogen fixation in cyanobacterial mats. In 1988 he became associated professor at the University of Amsterdam in the Department of Aquatic Microbiology. In 1996 he became head of the Department of Marine Microbiology of the Netherlands Institute of Ecology, which became part of the Royal Netherlands Institute of Sea Research in January 2012. In 2002 he was a Visiting Professor at the University Miguel Hernandez in San Juan de Alicante in Spain. From 2007 he has also a chair in Marine Microbiology at the University of Amsterdam. Lucas Stal is an expert in cyanobacteria ecology and physiology. He studied in particular the fixation of nitrogen by various types of cyanobacteria as well as in natural ecosystems such as cyanobacterial blooms and microbial mats. He published almost 200 research papers and reviews.

Acknowledgements

We thank the many colleagues who have contributed to this volume. Ashwani K. Rai and Naveen K. Sharma wish to thank their families, friends, and colleagues for their encouragement and support during the time we have been working on this book. Many thanks are due to Fiona Seymour, Senior Project Editor, Life Sciences Book Content Management, Wiley UK for all the support, input, and assistance she provided and for her patience until all the chapters were delivered.

About the book

Cyanobacteria are oxygenic photosynthetic autotrophs and are among the most successful and earliest forms of life we know. Globally, cyanobacteria are important primary producers and play a crucial role in the biogeochemical cycles of nitrogen, carbon, and oxygen. Cyanobacteria are recognized for their high potential in a large variety of biotechnological applications. They are used as food supplements in many countries all over the world. The exploitation of cyanobacteria as a source of a wide range of structurally novel and biologically active compounds that are valuable for drug development is attracting considerable and increasing interest from the pharmaceutical industry. From a biotechnological perspective, cyanobacteria are unique cell factories that combine the cost-effective energy-capturing ability of photosynthesis with high cultivation yields, which are desirable for an economic, industrial-scale production process. This volume is an authoritative and comprehensive overview of the current and future possibilities for industrial scale utilization of cyanobacteria. The book is divided into five parts and twenty-one chapters on various aspects of the biotechnological applications of cyanobacteria. Part I describes general characteristics and classification of cyanobacteria. Part II contains chapters on the ecological services rendered by cyanobacteria. Part III describes the exploitation of cyanobacteria and the currently unexplored potential of cyanobacteria. Part IV discusses the harmful aspects of cyanobacteria. The last part (Part V) includes topics on tools, techniques, and patents related to commercial aspects of cyanobacteria. This book is the first of its kind to provide an overview of the potential commercial exploitation of cyanobacteria and the opportunities and problems related to this exploitation. This book is meant as a useful resource for students, researchers and professionals in academia and the biotech industry.

Introduction

Naveen K. Sharma, Ashwani K. Rai, and Lucas J. Stal

At present, human society is confronted with serious issues related to environment, food, and energy (Tilman *et al.*, 2009). The burden on the environment in general and agricultural productivity in particular caused by the exponentially increasing population is phenomenal. In order to produce sufficient food for this massive human population, new ways and means have to be found that will increase food production substantially while taking into account the limits of the biosphere's ability to regenerate resources and provide ecological services. For this to happen, human society will rely on the huge potential of microorganisms to provide food, food additives, pharmaceuticals, and energy. Biotechnological applications for growing these microorganisms and exploiting their potential will make a huge leap in coming years. Cyanobacteria are of particular interest for biotechnological applications.

Cyanobacteria are Gram-negative oxygenic photosynthetic autotrophic organisms and are among the most successful and oldest forms of life (Schopf, 2000). Cyanobacteria enriched the primitive atmosphere with oxygen and therefore were crucial for the evolution of multicellular life. In an endosymbiotic event with a host cell, cyanobacteria became the origin of the chloroplast of the eukaryotic plant cell (Delwiche and Palmer, 1997). Cyanobacteria will occupy almost any illuminated habitat, ranging from aquatic to terrestrial environments as well as extreme habitats such as hot springs, hypersaline waters, deserts, and the polar regions. Several species are unique as they combine nitrogen fixation with oxygenic photosynthesis in a single organism. In Chapter 1, Aharon Oren gives an overview of the biology, ecology, and evolution of this fascinating group of microorganisms.

Cyanobacteria are a monophyletic but heterogeneous group of oxygen-evolving photosynthetic organisms. Cyanobacteria come in a remarkable variety of morphologies, including unicellular, colonial, filamentous, and branched filamentous forms, and their cell size may vary across two orders of magnitude, while the length of trichomes and the size of aggregates may be macroscopic and visible to the naked eye. The taxonomy of cyanobacteria is still the subject of debate. Although cyanobacteria obviously belong to the domain Bacteria, this does not solve the taxonomic problems, which have been classically based on the rich morphological characteristics. The phylogeny based on the DNA sequence of the 16S rRNA gene confounded the classical system. Many prefer the classical system because of the system of nomenclature and because of the recognition of species by microscopy. In Chapter 2, Jiří Komárek shares his views on cyanobacterial taxonomy.

During their long evolutionary history, cyanobacteria have undergone several structural and functional modifications, and these are responsible for their versatile physiology and wide ecological tolerance. The ability of cyanobacteria to tolerate high temperature, UV radiation, desiccation, and water and salt stresses contributes to their competitive success in a wide range of environments (Whitton 2012; Herrero and Flores, 2008). Cyanobacteria can photosynthesize at low light intensities and can use bicarbonate ion for carbon dioxide fixation at high pH. Many species fix atmospheric nitrogen and assimilate it as a source of nitrogen. Also, cyanobacteria can use a variety of different sulfur sources and possess efficient phosphate acquisition mechanisms that allow them to live

in low-phosphate environments (Sharma *et al*., 2011, and references therein).

Globally, cyanobacteria are important primary producers and play an important role in the biogeochemical cycles of nitrogen, carbon, and oxygen. It is estimated that cyanobacteria may be responsible for half of the global primary production of these gases. In Chapter 3 Beatriz Díez and Karolina Ininbergs have touched upon the ecological importance of cyanobacteria. The ongoing climatic change is threatening the existence of the human population. The increase in atmospheric carbon dioxide, especially since the industrial revolution, has led to global warming. Autotrophic life can play an important role in mitigating the problem of global change. Eduardo Jacob-Lopes and colleagues have discussed the importance of cyanobacteria in carbon sequestration in Chapter 4. One can easily visualize the luxuriant cyanobacterial growth on historically and culturally important monuments and buildings. Nitin Keshari and Siba Prasad Adhikari discuss various aspects of this phenomenon, including protection methods and the economics involved, in Chapter 5.

For a long time, the economic importance of cyanobacteria was limited to their use as bio-fertilizer in agriculture mainly because of their capacity to fix nitrogen. Technological progress has opened new avenues for the biotechnological applications of cyanobacteria and scientific researchers have made many new discoveries, leading to novel compounds and uses of these organisms. Consequently, many new areas of interest have emerged and a majority have enormous commercial potential. In Chapters 6–14 some of the commercially important products and processes are discussed, with emphasis on economic aspects. The chapters include: therapeutic molecules from cyanobacteria (Rathinam Raja and colleagues, Chapter 6); a detailed account of *Spirulina* as an example for the production of neutraceuticals by Masayuki Ohmori and Shigeki Ehira (Chapter 7); ultraviolet photoprotective compounds from cyanobacteria and their biomedical applications (Tanya Soule and Ferran Garcia-Pichel, Chapter 8); cyanobacteria as biofertilizers (Radha Prasanna and colleagues, Chapter 9); cyanobacteria as a source for the production of biofuels (Naveen K. Sharma and Lucas J. Stal, Chapter 10); the synthesis of cellulose by cyanobacteria (Milou Schuurmans and colleagues, Chapter 11); the production of exopolysaccharides by cyanobacteria (Giovanni Colica and Roberto de Philippis, Chapter 12); the production of phycocyanin from cyanobacteria (Ruperto Bermejo, Chapter 13); and cyanobacterial polyhydroxyalkanoates as an alternative source for biodegradable plastic (Shilalipi Samantaray and colleagues, Chapter 14).

While cyanobacteria provide a wide range of benefits, many species cause environmental and health problems and represent a nuisance to human society. There are many species of cyanobacteria that cause blooms in water bodies, both marine and freshwater, resulting in a loss of water quality and possible toxicity to aquatic life. In Chapter 15, David P. Hamilton and colleagues deal extensively with cyanobacterial blooming in freshwater bodies. Many bloom-forming cyanobacteria produce a range of secondary metabolites that are toxic to various life forms. These toxins (referred to as "cyanotoxins") affect the liver (hepatotoxin), the nervous system (neurotoxin), or the skin (dermatotoxin) and represent a serious hazard to human and animal health. Jason N. Woodhouse and colleagues explain the economic fallouts caused by cyanotoxins in Chapter 16.

Cyanobacteria are natural solar-powered cellular factories synthesizing an array of natural compounds useful for human welfare. We have just started to tap this resource. According to Pulz and Gross (2004) the microalgal biomass (including cyanobacteria) market has a size of about 5,000 t/year of dry matter accounting for around US$ 1.25×10^9/year (Table 1). However, authentic data on the cyanobacteria-based market is lacking. Industrial biotechnology involves conversion of biomass via biocatalysis, microbial fermentation, or cell culture to provide material, chemicals, and energy. It is cost-competitive, environment friendly, and sustainable.

Successful industrial-scale biotechnology mainly depends on getting a suitable organism with the desired property. Cyanobacteria could contribute greatly to this enterprise. Formulation of appropriate culture conditions and suitable culture media and their extrapolation to large-scale systems is essential for industrial success. Thus there is a need to combine metabolic engineering with advances in photobioreactor technology. A. Catarina Guedes and colleagues have described various large-scale culture systems

Table 1 Projected market estimates for microalgal (including cyanobacteria) products. The estimate excludes biofuels and other valuable services.

Sector	Product	Retail value US $ × 10^6/year)	Current status	Important genera
Biomass	Health food	1.25–2.5 × 10^9	Growing	*Chlorella*, *Spirulina* (*Arthrospira*) *Dunaliella*
	Functional food	800	Growing	*Spirulina*
	Feed additive	300	Fast-growing	*Chlorella*, *Scenedesmus*, *Spirulina*
	Aquaculture	700	Fast-growing	40 species, *Chlorella*, *Spirulina*, *Dunaliella*
	Soil conditioner	5 × 10^9	Promising	N_2 fixers
Coloring substances	Astaxanthin	<150	Starting	*Haematococcus pluvialis*
	Phycocyanin	>10	Stagnant	Cyanobacteria
	Phycoerythrin	>2	Stagnant	Cyanobacteria
Antioxidants	β-carotene	>280	Promising	*Dunaliella*
	Tocopherol	Unknown	Stagnant	*Spirulina*
	Antioxidant extract (CO_2)	100–150	Unknown	*Spirulina*
	Arachidonic acid	20	Growing	*Euglena, Porphyridium, Parietochlods*
	Docosahexaenoic acid	1.5 × 10^9	Fast-growing	*Crypthecodinium cohnii*
	Polyunsaturated fatty acids	10	Fast-growing	*Schizochytrium* resp. *Cryptecodinium*, *Spirulina, Ulkenia, Odontella aurita*
Other products	Toxins	1–3	Unknown	*Microcystis, Aphanizomenon*
	Isotopes	>5	Unknown	All

From Pulz and Gross, 2004.

including their pros and cons in Chapter 17. An example of large-scale industrial culturing of cyanobacteria has been provided by Hiroyuki Takenaka and Yuji Yamaguchi in Chapter 18.

Modern genetic techniques are capable of increasing the content of valuable products in a desired organism. Lack of cell differentiation and absence of allelic genes make unicellular cyanobacteria a simple system for genetic manipulation. However, hitherto the progress has been slow and suffers from drawbacks. Timo H. J. Niedermeyer and colleagues have described the various genetic manipulation techniques applicable to cyanobacteria in Chapter 19.

In Chapter 20, John G. Day has described the techniques of cryopreservation for long-term storage of useful cyanobacterial strains. It is necessary to move from an academic conception (relevant patents are discussed by Michael A. Borowitzka in Chapter 21) to industrial reality. Until now, with a few exceptions, which are discussed in this book, hardly any cyanobacteria products with potential commercial value have been successfully and economically produced at the industrial scale. The few industrial products that are available in the market include mainly health foods or other niche products. Nevertheless, favorable environmental and economic aspects and largely unharnessed potential make cyanobacteria a promising future resource (Sharma *et al.*, 2011). To make the production cost-effective a "biorefineries approach", which includes the simultaneous production of the main product with other side products, could be adopted.

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About the companion website

This book is accompanied by a companion website:

www.wiley.com/go/sharma/cyanobacteria

The website includes:
- Powerpoints of all figures from the book for downloading
- PDFs of all tables from the book for downloading

PART I

Biology and classification of cyanobacteria

Chapter 1

Cyanobacteria: biology, ecology and evolution

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1.1 Introduction

The first time I observed a prokaryotic microorganism through the microscope was during my first semester as a biology student in Groningen, the Netherlands, in the end of 1969. During the introductory botany course a young faculty member named Wytze Stam showed me filaments of *Anabaena* with many heterocysts, hidden within the leaf cavities of the water fern *Azolla* (see Adams, Duggan and Jackson, 2012 for more information). Later Wytze Stam became a pioneer of molecular systematics studies of cyanobacteria (then called "blue–green algae"), being the first to apply the technique of DNA–DNA hybridization to elucidate taxonomic relationships between different species (Stam and Venema, 1977).

I consider it a special privilege to have been invited to write the introductory chapter to *Cyanobacteria – an Economic Perspective*, considering the fact that I have never worked on economic and biotechnological aspects of cyanobacteria, and that during most of my career my studies concentrated on entirely different types of prokaryotes: anoxygenic phototrophic purple sulfur bacteria during my M.Sc. studies and, later, different groups of halophilic Archaea and Bacteria. Still, the cyanobacteria kept fascinating me, and during several periods of my life I have studied different aspects of this important group of prokaryotes. My Ph.D. studies in Jerusalem centered on the ability of certain cyanobacteria, and in particular a filamentous strain from Solar Lake, Sinai, designated *Oscillatoria limnetica*, to perform not only oxygenic photosynthesis, but also anoxygenic photosynthesis with sulfide as an electron donor, enabling the organisms to lead an anaerobic life (Garlick, Oren, and Padan, 1977; Oren, Padan, and Avron, 1977; Oren and Padan, 1978). The finding that some cyanobacteria also have well-developed modes of survival in the dark under anaerobic conditions, including fermentation and anaerobic respiration with elemental sulfur as electron acceptor (Oren and Shilo, 1979), showed how

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well certain members of the group are adapted to an anaerobic lifestyle.

During my later studies of microbial life at high salt concentrations and the adaptations of microorganisms to hypersaline conditions I developed an interest in solar saltern ponds for the production of salt. Along the salinity gradient in the evaporation ponds beautiful benthic microbial mats often develop, dominated by cyanobacteria. One of the most spectacular displays of cyanobacteria I know is within the crusts of gypsum that accumulate on the bottom of saltern ponds with salinities between 150 and 200 g/l: an upper orange-brown layer of *Aphanothece*-type unicellular species, then a bright dark-green layer of *Phormidium*-type filaments, below which a red layer of photosynthetic purple bacteria is found. This intriguing and very esthetical system became not only one of my favorite objects for research (e.g., Oren, Kühl, and Karsten, 1995; Oren *et al.*, 2008, 2009), but also a tool for teaching students about the nature of stratified systems and the influence of different physical and chemical gradients on microbial communities. A brief opportunity to study the microbiology of the hot springs (up to 63°C) on the eastern shore of the Dead Sea in Jordan extended my work on extremophilic cyanobacteria to the thermophiles as well (Ionescu *et al.*, 2009, 2010).

In recent years I became involved in an entirely different aspect of the cyanobacteria: problems connected with the systematics and in particular with the nomenclature of the group. In the course of my activity within the International Committee on Systematics of Prokaryotes I realized that the cyanobacteria are a highly problematic group as far as nomenclature is concerned. On the one hand they were traditionally considered to be plants and their nomenclature was therefore regulated by the provisions of the International Code of Botanical Nomenclature (since 2012: the International Code of Nomenclature for algae, fungi, and plants); on the other hand, they belong to the prokaryotic world and as such their nomenclature may be regulated by the International Code of Nomenclature of Prokaryotes (The Bacteriological Code) (Oren, 2004, 2011; Oren and Tindall, 2005). This led to interesting discussions with the cyanobacterial taxonomists (Oren and Komárek, 2010; Oren, Komárek and Hoffmann, 2009). No quick solution of the many remaining nomenclature problems can be expected in the near future.

Thinking about the invitation by the editors of this book to write a chapter entitled "Cyanobacteria – biology, ecology and evolution," it is clear that such an introductory chapter can never cover all aspects. I therefore chose to briefly highlight a number of the topics related to the life of the cyanobacteria that fascinate me most.

- Cyanobacteria have been around on our planet for a very long time and they were the first organisms to form molecular oxygen and to change the biosphere from anaerobic to largely aerobic.
- Cyanobacteria are a morphologically diverse group, more diverse than any other group of prokaryotes, and some show unique patterns of cell differentiation.
- Many cyanobacteria have a global distribution, and they are excellent model organisms to investigate questions of microbial biogeography and evolution.
- Cyanobacteria are major contributors to the primary production of the oceans, and they are one of the most important groups that fix molecular nitrogen.
- Cyanobacteria are highly efficient in adapting to their environment; many can actively move toward more favorable areas; they adapt their pigmentation according to the intensity and sometimes also to the color of the available light; some show surprising adaptation toward a life under anaerobic conditions; many types thrive at extremes of temperature, salinity, and pH; and when growth conditions are not suitable, some species can survive adverse conditions for long periods.
- Most types of cyanobacteria are relatively easy to grow in the laboratory, and many have been obtained and studied in axenic culture.

Because of my interest in the history of microbiology, I refer throughout the chapter to the historical aspects of the research, trying to show how different concepts and ideas have developed through time.

1.2 Cyanobacteria are ancient microorganisms

The Precambrian has been termed "the age of blue–green algae" (Schopf, 1974), and Schopf and Walter (1982) called the Proterozoic era – the period between

2.5 and 0.54 billion years (Ga) ago when the atmosphere turned from anoxic to oxygenated as a result of oxygenic photosynthesis – "the age of cyanobacteria."

Although there is still considerable controversy about the exact time the cyanobacteria started to appear on Earth, there is be no doubt that they are extremely ancient organisms. There is evidence that oxygenic photosynthesis occurred even in the Archean era (Knoll, 1979; Olson, 2006), possibly even >3.7 Ga ago (Rosing and Frei, 2004). The Precambrian sedimentary record abounds with microfossils that resemble different types of present-day cyanobacteria, and it is generally assumed that the cyanobacteria originated well before 2.5 Ga ago (Schopf, 1970, 1993, 2012; Schopf and Barghoorn, 1967; Schopf and Packer, 1987). Four key rock sequences are known that have survived without major changes in the metamorphosed state from the first billion years (3.8–2.8 Ga) of Archean Earth history:

- the Warrawoona and George Creek Groups of Western Australia, ~3.5 Ga old
- the Onverwacht and associated groups of southern Africa, ~3.5 Ga old
- the Pongola Supergroup of Natal, ~3.1 Ga old
- the Fortescue Group of Western Australia, ~2.8 Ga old (Schopf and Walter, 1982).

The oldest reliable microfossils are those from the Apex chert of northwestern Western Australia and the Fig Tree series of South Africa (3.1 Ga). Some of these, which may or may not have been cyanobacteria, have been referred to as alga-like (Pflug, 1967; Schopf and Barghoorn, 1967; Pflug *et al.*, 1969; Schopf, 1993). But one cannot be certain that such "alga-like" unicellular structures were indeed cyanobacteria.

Much has been written about the nature of the Precambrian stromatolites – layered rocks that resemble the properties of modern stratified microbial mat communities of cyanobacteria – and, since their discovery in the 1960s, the microfossils found in them (Barghoorn and Tyler, 1965; Cloud, 1965; Buick, 1992; Grotzinger and Knoll, 1999). There seems to be little doubt about the cyanobacterial nature of microfossils present in stromatolites of the Transvaal sequence (2.25 Ga) (MacGregor, Truswell, and Eriksson, 1974; Nagy, 1974), and biomarkers possibly derived from cyanobacteria (methylhopanoids – derivatives of 2-methylbacteriohopanepolyols – which occur in many modern species) have been found in organic-rich sediments as old as 2.5 Ga (Summons *et al.*, 1999). Altermann (2007) provided a critical discussion of the different reported claims for the finding of more ancient, 3.8–2.5 Ga-old fossils of cyanobacteria.

The modern stromatolites discovered in the late 1950s in Shark Bay, a slightly hypersaline marine lagoon in Western Australia (Logan, 1961), are often considered as equivalents of the fossil stromatolites that have remained from the Precambrian. These stromatolites have been studied in depth (Bauld, 1984; Stal, 1995, 2012), but it still cannot be ascertained to what extent the communities in Shark Bay indeed resemble the kind of structures built at the time oxygenic phototrophs first colonized the planet and started to release oxygen to the atmosphere.

1.3 Cyanobacteria are morphologically diverse

Cyanobacteria can be defined to include all known prokaryotes capable of oxygenic photosynthesis. Phylogenetically (as based on the small-subunit ribosomal RNA-based tree of life) they are a coherent group within the domain Bacteria (Bonen, Doolittle, and Fox, 1979; Wilmotte, 1994; Wilmotte and Herdman, 2001). The cyanobacterial lineage also includes the chloroplasts of the eukaryotic cells and plants (Giovannoni *et al.*, 1988).

Descriptions of cyanobacteria started appearing in the botanical literature from the end of the 18th century onwards. In the early times the group was generally referred to as "Schyzophytae," the name "Cyanophyceae" was introduced by Sachs in 1874 and "Cyanophyta" by Smith in 1938. The earliest described genus is probably *Rivularia* (Roth, 1797–1806); *Oscillatoria* and *Nostoc* were published in 1803 by Vaucher, who curiously placed these organisms in the animal kingdom (Fogg *et al.*, 1973). Many species of cyanobacteria were described in the monographs on algae by Lyngbye (1819), Agardh (1824), and Kützing (1845–1849), the first and third of which are beautifully illustrated. These and other 19th century books were the precursors of more recent morphology-based taxonomic treatises on the Cyanophyceae/Cyanophyta

by Geitler (1932) and Desikachary (1959). Morphologically the group is much more diverse than any other group within the prokaryotes, Bacteria and Archaea combined, so that taxonomic schemes are still largely based on morphological characters. However, molecular sequence information is becoming increasingly important in the classification of the cyanobacteria (Wilmotte, 1994; Wilmotte and Herdman, 2001).

The affiliation of the "blue–green algae" with the bacteria rather than with other groups of algae was realized by Ferdinand Cohn already in the second half of the 19th century (Cohn, 1872, 1875, 1897):

> Perhaps the designation of Schyzophytae may recommend itself for this first and simplest division of living organisms, which appears to me naturally delimited from the higher plants, even although its distinguishing characters are negative rather than positive.
> (Cohn, 1875; translation R.Y. Stanier)

After the fundamental division of the living organisms into prokaryotes and eukaryotes had become firmly established in the middle of the 20th century, it was time to re-evaluate the position of the "blue–green algae." In their classic essay entitled 'The concept of a bacterium', Stanier and van Niel (1962) made the following important statement (original emphasis):

> It is now clear that among organisms there are two different organizational patterns. . . the eucaryotic and the procaryotic type. *The distinctive property of bacteria and blue-green algae is the procaryotic nature of their cells.* It is on this basis that they can be clearly segregated from all other protists (namely, all other algae protozoa and fungi), which have eucaryotic cells. (Stanier and van Niel 1962. Reproduced with kind permission from Springer Science + Business Media.)

When, based on the new insights into the nature of the "blue–green algae," Roger Stanier and his colleagues a few years later formally proposed placing the nomenclature of the group under the rules of the International Code of Nomenclature of Bacteria (Stanier *et al.*, 1978), heated discussions started between the bacteriologists and the botanists on the issue of the nomenclatural system under which the group should be treated. Meeting sessions and even entire symposia were devoted to the question (Friedmann and Borowitzka, 1982; Castenholz, 1992; Oren, Komárek, and Hoffmann, 2009; Oren and Komárek, 2010). As explained above, the issue is still largely unresolved.

A recent attempt to classify the cyanobacteria primarily on the basis of morphological traits while incorporating as much "polyphasic" information as possible by including other characteristics was made by the editors and authors of the last edition of *Bergey's Manual of Systematic Bacteriology*, as outlined by Castenholz (2001). In this system, the unicellular types are grouped in Subsections I and II. Cyanobacteria in Subsection I divide by binary fission while those in Subsection II can also undergo multiple divisions. The purple-colored unicellular *Gloeobacter violaceus*, an organism that is unique as it is the only cyanobacterium known that lacks thylakoids (Rippka, Waterbury, and Cohen-Bazire, 1974) roots phylogenetically deeply with Subsection I. Cyanobacteria belonging to Subsection II can form a large number of very small daughter cells named baeocytes, which subsequently grow out to normal-sized cells. Some members of the *Pleurocapsales* (Subsection II) such as *Dermocarpa* and *Hyella* can reach very large cell sizes (up to 30 µm and more), particularly when they are about to divide multiple times to produce baeocytes. The filamentous cyanobacteria are grouped in Subsections III, IV, and V. Subsection III consists of filaments composed of one cell type (the *Oscillatoriales*). Subsections IV and V comprise filamentous cyanobacteria that exhibit cell differentiation, a rare phenomenon among prokaryotes. All representatives belonging to Subsections IV and V are capable of fixing nitrogen. Subsection V is characterized by true branching of trichomes, resulting from the division of cells in more than one plane, forming what may be the most advanced type of morphological structure attained in the prokaryote world.

1.4 Cyanobacteria as model organisms for microbial biogeography studies

Many species of cyanobacteria have a cosmopolitan distribution. An excellent example is the terrestrial *Nostoc commune*, found in both temperate, tropical, and polar regions, on the continents as well as on isolated islands. Some of these cosmopolitan types have become popular objects to test theories about

biogeography and microevolution. The famous statement by Lourens Baas Becking (1934) – "*Alles is overal*: maar *het milieu selecteert*" ("Everything is everywhere: *but*, the environment selects") – has been the starting point for several comparative studies of cyanobacterial populations in similar habitats worldwide. One of those species is the halophilic filamentous organism previously known as *Microcoleus chthonoplastes* and recently renamed *Coleofasciculus chthonoplastes* (Siegesmund *et al.*, 2008). An in-depth comparative phenotypic and phylogenetic analysis of material collected from disparate geographical locations only showed very slight differences, if at all (Garcia-Pichel, Prufert-Bebout, and Muyzer, 1996). A similar global dispersal without clear differences between geographically separated populations, as based on sequence comparisons of the ITS (internal transcribed spacer) region between the 16S and the 23S rRNA genes, was observed for the freshwater planktonic *Microcystis aeruginosa* (Van Gremberghe *et al.*, 2011). Metagenomic studies showed a remarkably low genomic diversity, with <1% nucleotide divergence in several genes, among geographically widely distributed populations and isolated strains of the marine unicellular nitrogen-fixing *Crocosphaera watsonii* (Zehr *et al.*, 2007).

Of special interest as a model for the study of the geographical distribution and evolutionary processes on a local scale are the cyanobacterial communities in hot springs. Distances separating thermal spring areas can be thousands of kilometers, and there are no obvious mechanisms to explain how thermophilic cyanobacteria may migrate between such areas. Yet, surprisingly similar types, unicellular as well as filamentous, are found in springs of similar temperatures and water chemistry worldwide, although thermophilic *Synechococcus* types are absent from the hot springs of Iceland, Alaska, and the Azores (Papke *et al.*, 2003). Based on the analysis of 16S and 23S rRNA and ITS sequences, small geographical differences were found that could not be attributed to differences in the chemical properties of the spring waters. Genetic drift caused by geographical isolation was therefore postulated to be in part responsible for the observed evolutionary divergences (Papke *et al.*, 2003). More recent genetic analyses of the cyanobacterial communities in hot springs near the eastern shore of the Dead Sea in Jordan have extended these observations of global general similarities combined with minor local differences (Ionescu *et al.*, 2010).

1.5 Cyanobacteria are major contributors to the primary production in the oceans

In their monograph on *The Blue–Green Algae*, published in 1973, Fogg *et al.* wrote: ". . . except for *Trichodesmium*, which often forms massive blooms in tropical seas, blue–green algae are generally absent from the open sea. Why this should be so is difficult to explain at present." Thus at the time it was still generally assumed that oxygenic prokaryotes contribute very little to the productivity of the oceans on a global scale.

Today it is clear that a significant part of the carbon dioxide fixation in the oceans can be attributed to the activity of unicellular cyanobacteria, mostly organisms belonging to novel types discovered only in the past few decades. Some of these differ in pigmentation from the classic cyanobacterial pattern, which is characterized by chlorophyll *a* and phycobiliproteins as the main photosynthetic pigments. The first indications of the importance of small unicellular cyanobacteria in the plankton of the oceans came from electron microscopic studies by Sieburth and coworkers in the late 1970s (Johnson and Sieburth, 1979 and references therein) and from the observation of numerous orange-autofluorescent cells of phycoerythrin-rich cyanobacteria of the genus *Synechococcus* in the North Atlantic by Waterbury *et al.* (1979). *Synechococcus* is now known as a major contributor to the oceanic primary production (Scanlan, 2012). This type of *Synechococcus* has been isolated (Waterbury *et al.*, 1979). Some strains display an unusual type of swimming motility, the mechanism of which is still unclear (Waterbury *et al.*, 1985). The finding of *Prochlorococcus*, another member of the oxygenic picoplankton, containing divinyl chlorophyll *a* and *b* and lacking phycobilisomes (Chisholm *et al.*, 1988, 1992; Partensky, Hess, and Vaulot, 1999; Post, 2006; Scanlan and West, 2002) showed that the cyanobacteria may contribute even more to global carbon dioxide

fixation. More recently we learned about the existence of the chlorophyll *d*-containing *Acaryochloris* and the nitrogen-fixing *Crocosphaera watsonii*, all organisms widely distributed in the world's oceans (Waterbury, Watson, and Valois, 1988; Zehr *et al*., 2007). These discoveries have changed our conceptions of oceanic primary production (Paerl, 2012). Carr (1999) nicely summarized this when he wrote:

> The discovery about twenty years ago that a significant proportion of the primary production of the open oceans was driven by hitherto unrecognized unicellular cyanobacteria was of great moment to biological oceanography and extended the global role of these organisms by a vast scale.

The discovery of *Prochlorococcus* as a member of the cyanobacteria that contains a derivative of chlorophyll *b* was preceded by the recognition of other "Prochlorophyta" in aquatic environments. The first was *Prochloron*, a yet uncultured symbiont of marine ascidians (Lewin, 1975, 1977; Lewin and Withers, 1975). A decade later came the publication of the discovery of *Prochlorothrix hollandica*, a filamentous oxygenic photoautotrophic prokaryote containing chlorophyll *a* and *b*, from a freshwater lake in the Netherlands (Burger-Wiersma *et al*., 1986; Burger-Wiersma, Stal, and Mur, 1989). When then the unicellular *Prochlorococcus* was found as a major component of the picoplankton in the photic zone of the oceans (Chisholm *et al*., 1988; Partensky, Hess, and Vaulot, 1999), it became clear that such cyanobacteria with unusual types of chlorophyll are widespread. At one point it was proposed that the "Prochlorophyta" should be separated from the cyanobacteria as a separate phylum, but this is not justified on the basis of molecular phylogenetic, 16S rRNA-based studies: *Prochloron*, *Prochlorothrix*, and *Prochlorococcus* are three independent lineages of cyanobacteria. *Prochlorococcus* has unique divinyl-chlorophyll *a* and divinyl-chlorophyll *b* photopigments and only minor amounts of phycobiliprotein pigments. *Prochlorothrix* and *Prochloron* are the only prokaryotes known to possess chlorophyll *b* (Kühl, Chen, and Larkum, 2007).

The unicellular chlorophyll *d*-containing *Acaryochloris* was discovered during an attempt to isolate *Prochloron* from didemnid ascidians (Miyashita *et al*., 1996, 1997, 2003). The long-wavelength absorption peak of chlorophyll *d* is shifted to the red by about 30 nm as compared to chlorophyll *a*, showing an *in vivo* absorption peak at 710–720 nm. *Acaryochloris* does not contain phycobilisomes, but some phycocyanin and allophycocyanin may be present to function in light harvesting. Phylogenetically it does not show a close relationship to any of the other subgroups of unicellular cyanobacteria. In the ascidians it does not grow as a symbiont within the animal but rather forms dense cell patches in biofilms growing below the ascidian. It was also found as small epiphytic patches on a red macroalga. *Acaryochloris* is highly light-adapted and does not suffer from photoinhibition at high irradiance levels (Partensky and Garczarek, 2003; Kühl *et al*., 2005; Kühl, Chen, and Larkum, 2007). Another chlorophyll *d*-producing cyanobacterium related to *Acaryochloris* was isolated from the hypersaline Salton Sea of California, and was shown to harbor an unusual hybrid proteobacterial/cyanobacterial small-subunit rRNA gene (Miller *et al*., 2005).

1.6 Cyanobacterial nitrogen fixation – different strategies

Cyanobacteria are of great importance in the global nitrogen cycle as one of the major groups of prokaryotes capable of fixing gaseous nitrogen. The process is performed by many different types, unicellular as well as filamentous, with and without heterocysts (Stewart, 1980). Although the connection between cyanobacteria and biological nitrogen fixation was suspected for a long time, the first real proof that nitrogen is fixed was published only in 1969 for a unicellular representative (*Gloeocapsa* sp.) (Wyatt and Silvey, 1969). The presence of nitrogenase in heterocysts was documented in the same year (Stewart, Haystead, and Pearson, 1969).

Nitrogenase, the enzyme complex responsible for the reduction of nitrogen to ammonium ions, is irreversibly inactivated by molecular oxygen, the product of oxygenic photosynthesis. Therefore the two processes are basically incompatible. Various strategies have evolved in different groups of cyanobacteria to overcome this problem. One is based on spatial separation of the nitrogen-fixation process and oxygenic

photosynthesis; a second strategy is temporal separation: oxygen production during daytime and nitrogen fixation at night; it was recently discovered that some unicellular marine cyanobacteria may be altogether unable to evolve oxygen, and thus can maintain an active nitrogenase system. Even today the diverse mechanisms used by different types of cyanobacteria to combine nitrogen fixation with an aerobic mode of life are not completely clear.

The function of heterocysts, empty-looking thick-walled cells within filaments of pigmented cells, has for a long time baffled scientists, who called these structures "botanical enigmas" (Fritsch, 1951). In 1968 the role of the heterocyst as the site of nitrogen fixation could still be questioned (Fay *et al*., 1968): up to that time some workers considered the heterocysts as some kind of spores, dormant cells that allegedly were able to germinate under suitable conditions (Wolk, 1965). However, by 1853 Ferdinand Cohn had made a clear statement that these special cells cannot develop into new filaments:

> Nach diesen Beobachtungen scheint bei Anabaena, nicht wie bei Nostoc verrucosum nach Thuret, der ganze vielzellige Faden sondern jede einzelne vegetative Zelle, desselben im Stande zu sein, zu einem neuen Fadenhaufen sich zu vermehren, während die Dauerzellen keiner weiteren Entwicklung fähig sind. [According to these observations it is clear that in *Anabaena*, unlike in *Nostoc verrucosum* (Thuret), not the entire multicellular filament, but every single vegetative cell is able to multiply and develop into a new mass of filaments, while the resistant cells are incapable of further development] (Cohn, 1853).

Today we know that nitrogenase is located in the heterocyst, that these cells have lost the capacity of oxygenic photosynthesis and carbon dioxide fixation, but have retained photosystem I to supply the energy required for the nitrogen-fixation process. Respiration and oxidative phosphorylation also occur, so that nitrogen fixation does not strictly depend on light. The thick cell envelope serves as a gas diffusion barrier, providing a certain degree of protection of the nitrogenase against inactivation by oxygen (Haselkorn, 1978; Wolk, 1988). In *Anabaena*, heterocyst differentiation is controlled by a small diffusible peptide (Yoon and Golden, 1998). Heterocysts are found in Subsections IV and V (see the above section on morphological diversity). Heterocystous types are often found in symbiotic associations between cyanobacteria and eukaryotic organisms, based on the nitrogen-fixing ability of the prokaryotic partner. The *Azolla–Anabaena* symbiosis mentioned at the beginning of this chapter is a well-known case. Even more unusual is the intracellular occurrence of *Richelia intracellularis*, short filaments with a large terminal heterocyst, inside large marine pennate diatoms of the genera *Rhizosolenia* and *Hemiaulus*.

Not all nitrogen-fixing cyanobacteria produce specialized differentiated cells to harbor the nitrogenase. Temporal separation of photosynthetic oxygen production and nitrogen fixation is another strategy, used both by filamentous and unicellular species. When grown in media devoid of bound nitrogen under alternating light–dark cycles, an *Oscillatoria* sp. fixes nitrogen in the dark periods (Stal and Krumbein, 1985). A similar phenomenon was documented in the marine unicellular cyanobacterium *Crocosphaera watsonii* (Waterbury, Watson, and Valois, 1988).

Although it had already been observed more than half a century ago that nitrogen fixation in tropical seas is often associated with the presence of the filamentous non-heterocystous cyanobacterium *Trichodesmium* (Dugdale, Menzel, and Ryther, 1961), the properties of this organism are still to a large extent enigmatic: it lacks heterocysts but fixes nitrogen only in the light (Capone and Carpenter, 1982; Capone *et al*., 1997; Zehr *et al*., 1999). *Trichodesmium* blooms consist of long filaments that cluster to form aggregates of different morphologies. These aggregates can move great distances through the water column, their buoyancy being regulated by presence of gas vesicles (see below). It was previously hypothesized that the aggregates allowed interior cells to act as "heterocysts" (Carpenter and Price, 1976), but this does not appear to be the case. There are indications that nitrogenase is present in a subset of the cells in the trichome, termed "diazocytes" (Fredriksson and Bergmann, 1997). However, it is uncertain whether such diazocytes are terminally differentiated cells like the heterocysts. More likely, these cells turn temporarily into nitrogen-fixing cells. Therefore this strategy has been considered as a combination of spatial and temporal separation (Berman-Frank *et al*., 2001). Nitrogen fixation starts at the end of the night and lasts until midday. Thereafter, nitrogenase activity declines and the enzyme is rapidly and irreversibly inactivated in

the dark. The nitrogen-fixing cells are kept anoxic by a high rate of respiration. The relatively low solubility of oxygen in warm seawater may limit the geographic distribution of *Trichodesmium* to oceanic areas with water temperatures above 25°C. Under such conditions heterocysts are not required (Staal, Meysman, and Stal, 2003).

Even more curious is the recent discovery of unicellular cyanobacteria, abundant in tropical and subtropical oceans, that perform nitrogen fixation during the day. Their occurrence was first reported in the subtropical north Pacific Ocean in 2001 (Zehr *et al.*, 2001). These cells lack photosystem II and are thus incapable of oxygenic photosynthesis. Analysis of their genome indicates that they lack the enzymes of the Calvin cycle, the tricarboxylic acid cycle, and the pathways for the biosynthesis of some amino acids. These organisms have not yet been isolated, and they probably depend on some form of organic carbon provided by as yet unidentified other members of the marine ecosystem. Indeed most cyanobacteria are obligate photoautotrophs (Smith, London, and Stanier, 1967), but facultative photoheterotrophy and even chemoheterotrophy has been demonstrated in some species (Rippka, 1972). Thus there are precedents for heterotrophic growth of cyanobacteria, at least in laboratory culture. The interrelationships between this new form of marine life and the other organisms present in the oceanic waters are still entirely unknown (Zehr *et al.*, 2008; Tripp *et al.*, 2010; Bothe, Tripp, and Zehr, 2011).

1.7 Cyanobacteria use different strategies to move to areas where conditions are most favorable

Many cyanobacteria have the ability to move and to position themselves within a water column or within a benthic microbial mat so that conditions of light, nutrients, and other environmental parameters are the best achievable. There are basically two methods to reach this goal: passive movement based on the regulation of the buoyancy of the cells, and active motility at the expense of energy.

The presence of "gas vacuoles" – clusters of gas vesicles – in some cyanobacteria was already known in the 19th century. The first reports probably date from 1895, when three articles documented the buoyancy of different aquatic cyanobacteria and the sensitivity of the gas vesicles to pressurization (Ahlborn, 1895; Klebahn, 1895, Strodtmann, 1895). Gas vesicles are hollow cylinders built of protein subunits. The main protein that composes the vesicles (GvpA) is extremely hydrophobic, so that the inner side of the vesicle wall strongly repels water. A second protein, GvpC, strengthens the structure of the vesicles at the outer side. Gasses diffuse freely through the wall of the vesicles, so that these become filled with a gas mixture that reflects the composition of the gasses dissolved in the surrounding water. The number of gas vesicles per cell can be regulated to increase or decrease the buoyancy of the cells (Walsby, 1981, 1987, 1994). Among the genera whose distribution in aquatic environments is strongly dependent on the gas-vesicle content of the cells one can mention *Microcystis* and *Anabaena*, which often form dense blooms in nutrient-rich lakes, and *Trichodesmium*, mentioned above as one of the main marine nitrogen-fixing organisms. The large number of gas vesicles at the periphery of the *Trichodesmium* cells and the occurrence of the buoyant filaments in large bundles allow the masses of red, phycoerythrin-rich patches of cyanobacteria to float to the sea surface.

Active movement by cyanobacteria is possible as well. Many species display gliding movement: self-propulsion over a solid substrate or along other trichomes. Gliding movements are slow, generally in the order of 1–10 μm/s. Genera noted for their conspicuous movements are *Oscillatoria* and *Spirulina*, gliding being accompanied by the rotation of the trichome along its axis. The direction of movement can be triggered by gradients of environmental parameters, notably the intensity and quality of the light available, but other factors can be important as well, including even the salinity of the medium. Thus, "halotaxis," where cells move in the direction of optimal salt concentrations, was recently identified in the cyanobacterial community in an intertidal hypersaline microbial mat (Kohls *et al.*, 2010).

Gliding movement is strictly dependent on the presence of a solid substratum. Occurrence of flagella, being the most common mode of active movement in the world of prokaryotes, has not yet been documented

for cyanobacteria. Therefore, the discovery of marine unicellular *Synechococcus* cells that actively swim in the water, without the requirement for a solid surface, came as a great surprise (Waterbury *et al*., 1985). Electron microscopic examination did not reveal the presence of flagella or any other cellular structures connected with locomotion. The mode of swimming motility of these marine planktonic organisms is poorly understood and its function is obscure (Brahamsha, 1996).

1.8 Some cyanobacteria change color as a function of light quality

Cyanobacteria can be many different colors: green, blue–green, brown, purple, red, and even almost black. The color of the cells, and accordingly the range of wavelengths absorbed by the photosynthetic apparatus, depends on the types of pigments present and the amounts and ratios in which these pigments are found. A pigment common to nearly all is the green chlorophyll *a*, which is the pigment of the reaction centers of both photosystem I and photosystem II. Exceptional cases such as *Prochlorococcus* and *Acaryochloris* were discussed earlier. Different types of carotenoids (echinenone, myxoxanthophyll, and others) are commonly present as well. And, with very few exceptions, cyanobacteria have phycobilisomes attached to the thylakoid membranes. The phycobilisome is a macromolecular complex composed of phycobiliproteins, which can constitute up to 40% of the soluble cellular protein. Allophycocyanin (maximum absorbance 650 nm) is found in the core substructure, the part of the phycobilisome that is in direct association with the photosynthetic reaction center. The blue phycocyanin (absorption peak 620 nm), the red phycoerythrin (565 nm) and the comparatively rare phycoerythrocyanin (570 nm) are present in rods that emanate from the core. Two subunit forms (α and β) exist of each of these phycobiliproteins, and these are assembled as heterodimers into higher-order structures; these aggregates are the building blocks of the phycobilisome (Bazire, 1971; Glaser and Cohen- Glaser, 1983; Grossman *et al*., 1993; Carr, 1999; Stal, 2007).

Certain species, notably strains of *Oscillatoria* and *Phormidium*, change their color as a function of the spectral properties of the light in which they are grown. The phenomenon is known as "complementary chromatic adaptation." It was first observed by Theodor Wilhelm Engelmann in 1883. When the organisms are illuminated with a spectrum of light, they assume, over the course of a day or two, a color complementary to that part of the spectrum in which they are growing. Engelmann therefore suggested that the pigmentation is usually such as to permit the most efficient use of the wavelengths available in the particular habitat in which the cells grow. Complementary chromatic adaptation was studied intensively in the first decades of the 20th century (Engelmann, 1902; Gaidukov, 1902; Boresch, 1922). Today we know that the phenomenon is caused by a change in the ratio in which the different phycobiliproteins are incorporated into the rods of the phycobilisomes. When the cells are exposed to red light, more blue phycocyanin is produced; when illuminated with green light, the red phycoerythrin is preferentially synthesized. The result is the creation of a new form of phycobilisome that is capable of more efficient harvesting of the most prevalent wavelengths of light in the environment (Tandeau de Marsac, 1977, 1983; Wyman and Fay, 1987; Kehoe and Grossmann, 1999).

1.9 A facultative anaerobic lifestyle is common in many cyanobacteria

In essence, the cyanobacteria lead an aerobic life, and, being oxygenic phototrophs, they increase the oxygen concentration of their environment in the light. However, different types, especially species of subsection III morphology, are found in abundance in environments such as layered microbial mats and stratified lakes where they are exposed to anaerobic conditions, either on a temporal basis (anaerobiosis at night, presence of oxygen, and possibly even oxygen supersaturation during daytime) or permanently in habitats such as sulfur springs or the monimolimnion of shallow meromictic lakes. Solar Lake, Sinai, is a prime example of an environment where cyanobacteria are found in oxygen-free conditions for prolonged periods (Cohen,

Padan and Shilo, 1975; Padan, 1979a; Padan and Cohen, 1982). Another intriguing ecosystem where cyanobacteria occur at low oxygen concentrations and in the presence of sulfide is the microbial mat system recently discovered in a sinkhole at a depth of 23 m at the bottom of Lake Huron, Michigan (Voorhies *et al*., 2012).

The first report on carbon dioxide assimilation by certain cyanobacteria in the presence of sulfide and an apparent absence of molecular oxygen dates from more than 70 years ago (Nakamura, 1938). However, the ability of some species to perform anoxygenic photosynthesis using sulfide instead of water as the electron donor for carbon dioxide photoassimilation and photoautotrophic growth was first unequivocally demonstrated in the 1970s in studies of the biota of Solar Lake (Cohen, Padan, and Shilo, 1975; Garlick, Oren, and Padan, 1977; Oren, Padan and Avron, 1977; Oren and Padan, 1978; Padan, 1979b). In the presence of sulfide, photosystem II and its water-splitting system does not function, and electrons are fed from sulfide directly to the donor site of photosystem I. This type of anoxygenic photosynthesis may be active in cyanobacteria in stratified microbial mats that are often exposed in the morning hours to high concentrations of sulfide as a result of bacterial sulfate reduction during the night. Later, when the sulfide is depleted, the cells shift to conventional oxygenic photosynthesis. A (partially) anaerobic lifestyle can also be reflected in the lipid metabolism of such cyanobacteria: polyunsaturated fatty acids, the synthesis of which is oxygen-dependent (Kenyon and Stanier, 1970), are lacking, and the "bacterial," oxygen-independent pathway can be used for the biosynthesis of monounsaturated fatty acids (Oren *et al*., 2009).

Adaptation of such facultative anoxygenic cyanobacteria to life under anaerobic conditions is not limited to the daylight hours. When aerobic respiration is not possible in the hours of darkness, alternative ways of energy generation must be operative. At least two such modes of obtaining energy have been identified. In some cases, elemental sulfur, accumulated during daytime, for example as the oxidation product of sulfide in the process of anoxygenic photosynthesis, can serve as the electron acceptor for the oxidative breakdown of intracellular storage polymers formed during the daytime (Oren and Shilo, 1979). Another way to obtain energy in the dark when neither oxygen nor alternative electron acceptors are available is by fermentation. Intracellular glycogen-like storage material can be fermented to products such as lactate and acetate with the gain of ATP (Oren and Shilo, 1979; Heyer, Stal and Krumbein, 1989; Stal and Moezelaar, 1997). Virtually all species of cyanobacteria that grow in microbial mats are capable of fermentation (Stal and Moezelaar, 1997).

1.10 Cyanobacteria have adapted to life in extreme environments

"Patavinorum aquis calidis herbae virentes innascunutur. . . " ["Green grass grows in the hot springs of Padua. . . "]: thus wrote Pliny the Elder in Book II of his *Natural History*. The growth of cyanobacteria like "green grass" in the hot springs near Padua can still be observed today, proving that some cyanobacteria are adapted to life in extreme environments. The studies by Tom Brock and his colleagues in Yellowstone National Park, USA (Castenholz, 1969; Brock, 1978; Ward, Castenholz and Miller, 2012) and further studies of the microbial communities inhabiting hot springs all over the world have defined the upper temperature limit for photosynthesis at 73–74°C. The unicellular cyanobacterium known as *Synechococcus lividus* or *Thermosynechococcus* (a species name still without standing in the botanical and in the bacteriological nomenclature) is the most thermotolerant; the filamentous *Mastigocladus* type starts appearing at somewhat lower temperatures. Curiously, a study of the cyanobacteria of Yellowstone National Park performed in the early 1930s documented apparent cyanobacterial growth at much higher temperatures: an upper limit of 85.2°C was found for two newly described members of the Oscillatoriales: *Phormidium bijahense* and *Oscillatoria filiformis*, observed in a spring identified as "Meadow Spring." Members of the *Chroococcales* were then found at Yellowstone up to 84°C and *Chamaesiphonales* up to 80°C (Copeland, 1936).

As already mentioned above in the discussion of the stratified communities of cyanobacteria within gypsum crusts on the bottom of saltern evaporation

ponds at salinities of 200 g/l and above (Oren, Kühl, and Karsten, 1995; Oren *et al.*, 2009), and in the discussion of the cyanobacterial growth in the depths of Solar Lake, Sinai, with up to 180 g/l salt (Padan, 1979a; Padan and Cohen, 1982), some members of the group are also adapted to life at high salt concentrations. The first description of cyanobacteria living in concentrated brines was probably that by Trijntje Hof and Pierre Frémy (1933). We know of unicellular as well as filamentous halophilic or highly halotolerant representatives (Brock, 1976; Garcia-Pichel, Nübel, and Muyzer, 1998; Oren, 2012). However, heterocystous types appear to be absent from high-salt environments. Halophilic cyanobacteria accumulate organic "compatible" solutes to provide osmotic equilibrium between the cytoplasm and the outside medium. Glycine betaine (trimethylglycine) is the commonly found osmotic solute in the most halotolerant types, while glucosylglycerol is accumulated by many somewhat less halophilic representatives (Oren, 2012). At the highest salt concentrations (halite saturation, >300 g/l salt) cyanobacteria are not successful, and the niche of an oxygenic phototroph–primary producer is occupied by eukaryotic unicellular green algae of the genus *Dunaliella*.

We also find cyanobacteria in abundance in cold environments such as lakes in Antarctica and in cold as well as in hot deserts, where low/high temperature stress is combined with drought stress (Vincent, 2007; Hu, Gao, and Whitton, 2012; Quesada and Vincent, 2012). Cyanobacteria also thrive in many alkaline lakes, and some tolerate both high pH and high salinity.

In view of the high degree of adaptability of the cyanobacteria as a group to different extremes of physical and chemical parameters, it is surprising to note that they are not very successful in colonizing acidic environments. The lower pH limit for their growth appears to be around 4–5, and they are seldom encountered between pH 5 and 6 (Brock, 1973). Oxygenic photosynthesis at lower pH values, combined with elevated temperatures, appears to be the specialty of the alga *Cyanidium* and related organisms. The occasional finding of planktonic picocyanobacteria of <2 μm in diameter at pH values down to 4.5 and the observation of several filamentous forms with true branching at about pH 4.0 are generally considered to set the lower pH limit for cyanobacteria. Occasionally there have been reports of cyanobacteria at even lower pH values: populations of two types of filamentous cyanobacteria resembling *Oscillatoria*/*Limnothrix* and *Spirulina* sp. were found in acidic lakes in Bavaria down to a pH of 2.9. In a survey of hundreds of lakes in Sweden and Canada cyanobacteria were generally present down to pH 3.7. *Aphanocapsa* and *Chroococcus*-like unicellular forms were found in acidified Canadian lakes (Steinberg, Schäfer, and Beisker, 1998).

1.11 Cyanobacteria can survive adverse conditions for prolonged periods

Water is essential to support all forms of life, but some cyanobacteria have a remarkable ability to survive for long periods in a desiccated state, to resume activity the moment water becomes available again (Potts, 1994, 1996). Their success as a major component of the biota of deserts, hot as well as cold ones (Wynn-Williams, 2000; Hu, Gao, and Whitton, 2012) is to a large extent due to their ability to survive desiccation. Other survival strategies against desiccation are the endolithic life of some species in protected areas below the surface of rocks (Friedmann, 1982) and the establishment of symbioses with fungi to form lichens. Most of the approximately 17,000 species of lichen known have green algae as the phycobiont, but cyanobacteria occur in about 8% of species. *Nostoc* is the most common cyanobacterium encountered in lichen symbioses, but *Stigonema*, *Scytonema*, *Calothrix*, *Dichothrix*, *Gloeocapsa*, *Chroococcus*, and *Hyella* have also been found (Ahmadjian, 1967).

Some types of filamentous cyanobacteria can, under adverse conditions, produce specialized cells called akinetes, which can remain dormant for years, to germinate and give rise to the development of new filaments when conditions become suitable again (Nichols and Adams, 1982; Herdman, 1987). Akinetes, sometimes termed "spores," are one of the two types of differentiated cells known in the world of cyanobacteria, the second being the heterocysts that fix molecular nitrogen, as discussed above. Akinetes do not survive exposure to high temperature like endospores of e.g., *Bacillus* and *Clostridium* spp. Akinetes are usually much larger than vegetative cells, and they

are only found in heterocystous cyanobacteria. They have been reported in members of the *Nostocaceae*, the *Rivulariaceae*, and in some species of *Stigonemataceae*. Thus in *Hapalosiphon* and *Fischerella*, vegetative cells may develop into such perennating structures. Production of akinetes has been known for more than 150 years: Carter (1856) drew filaments of *Rivularia* showing a large granular cell (which he called a sporangium), which always developed next to a heterocyst; germination experiments with akinetes were already being performed nearly a century ago (Harder, 1917a).

1.12 Many cyanobacteria readily adapt to cultivation in the laboratory

Most of our knowledge of the morphological diversity of cyanobacteria is based on the study of material collected from nature. However, growth conditions may have a strong impact on the morphological attributes of cyanobacteria, and the true diversity may thus be overestimated when only morphological criteria of field-collected material are examined. Stanier and coworkers (Stanier *et al.*, 1971; Rippka *et al.*, 1979; Rippka, Waterbury, and Stanier, 1981) called for the use of pure (axenic, not only free of other types of cyanobacteria but also devoid of contaminating heterotrophic bacteria) cultures for taxonomic studies. But to bring cyanobacteria in pure culture and adapt them to life under laboratory conditions is often extremely difficult. Growth media are simple, as they are based on inorganic salts and nutrients only (Allen, 1952; Waterbury, 2006), but this does not imply that all cyanobacteria are easy to grow and to maintain in axenic culture. Many types grow very slowly, and there may also be situations in which certain species depend on the presence of "contaminating" bacteria for growth.

The first axenic cultures of cyanobacteria were probably already established nearly a century ago. A strain of *Nostoc punctiforme* was obtained from a symbiotic association with a higher plant (*Gunnera* sp.) (one of the many documented cases of symbioses between cyanobacteria and eukaryotic organisms), and was shown to grow in bacteria-free culture in the dark on various sugars and polysaccharides (Harder, 1917b). Allison, Hoover, and Morriss (1937) reported that a strain of *Nostoc muscorum* grew slowly on glucose or sucrose in the dark, both in the presence and absence of combined nitrogen. However, Stanier, in his foreword to the monograph by Carr and Whitton (1982), attributed the first pure culture studies of cyanobacteria to Emerson and Lewis (1942), who grew an axenic culture of *Chroococcus* prepared by Klara Gaffron, antedating the first axenic *Synechococcus* sp. (*Anacystis nidulans*) of Allen (1952), probably the first axenic isolate still available today.

The large collection of axenic cultures of cyanobacteria of different types established by Stanier's group forms the basis of the extensive collection of cyanobacteria maintained in the Pasteur Culture Collection in Paris.

1.13 Final comments

The paragraphs above contain no more than a few highlights, showing different aspects of the fascinating group of the cyanobacteria. The selection of the topics presented is entirely personal, but I hope that the material covered is broad enough to provide a general introduction to the nature of the cyanobacteria, whose applied aspects form the subject of most chapters in this book. In an introductory chapter of similar nature my late friend Noel Carr (1999) wrote:

> . . . my first response will be some kind of general disclaimer with respect to synoptic balance or indeed total accuracy . . . The best that one can hope for is a personal, hopeful dispassionate, version of some of the developments and constraints that have happened in our own field . . .

The same disclaimer applies to this chapter as well. More in-depth general information on the world of the cyanobacteria can be found in earlier published monographs: books such as those by Bryant (1994), Carr and Whitton (1982), Fay and Van Baalen (1987), Fogg *et al.* (1973), and Whitton (2012) provide a wealth of useful information, as do many review articles (e.g., Cohen and Gurevitz, 2006; Stal, 2007; Stanier and Cohen-Bazire, 1977).

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Chapter 2
Modern classification of cyanobacteria

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2.1 Introduction

Cyanobacteria are one of the most interesting and important groups of autotrophic microorganisms from the evolutionary, phylogenetic, and ecological points of view. They originated in the early Precambrian (Schopf, 1974; Knoll and Golubić, 1992; Schopf, 1993; Pennisi, 1994; Schopf, 1996, 2000; Knoll, 2008), and remained for hundreds of millions of years as the only oxygenic photosynthesizing organisms in the Earth's biosphere. Thus, they have played an important role in the development of the oxygenated atmosphere of our planet (Tomitani, Duggan and Adam, 2006). One of the most important metabolic and structural adaptations in cyanobacteria is their ability to precipitate calcium carbonate in sheaths (Golubić, 1973; Golubić and Campbell, 1981; Merz, 1992; Pentecost, 1990, 2005; Golubić *et al.*, 2008). This is a property that might have assisted during their fossilization. Cyanobacteria were important travertine and stromatolite-forming organisms in the past, and contributed greatly to limestone deposits around the world.

The composition of the photosynthetic pigments in cyanobacteria is variable. Chlorophyll *a* and phycobiliproteins (phycocyanin, phycoerythrin, allophycocyanin) are the major pigments in cyanobacteria. However, some species also contain chlorophyll *b* or its derivatives (*Prochloron*, *Prochlorococcus*, *Prochlorothrix*), or chlorophyll *d* (*Acaryochloris*). There is large variability in the presence and ratio of phycobiliproteins (Castenholz, 2001). Some cyanobacteria are capable of changing the ratio of different phycobiliproteins in response to the prevailing light spectrum (i.e., chromatic adaptation). Different types of chromatic adaptation have been suggested (Tandeau de Marsac and Houmard, 1993; Bryant, 1994; Kehoe and Grossmann, 1999; Everroad and Wood, 2006). It is supposed to result from horizontal exchange of genes (Ong and Glazer, 1988; Palenik, 2001; Six *et al.*, 2007; Dufresne *et al.*, 2008), eventually leading to

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the development of fixed ratios of the blue and red pigments (cf. Kováčik *et al*., 2011).

Cyanobacteria possess remarkable adaptations to a large variety of habitats. They colonize various natural, human-managed ecosystems as well as extreme ecosystems such as hot and cold deserts, hypersaline environments, and hot springs with temperatures above 70°C (Peary and Castenholz, 1964; Castenholz, 1977; Ward and Castenholz, 2000). Reversible gas vesicles fasciculated in aerotopes develop within the cells of planktic populations and enable them to control their vertical movement in water reservoirs. These planktic types in particular produce specific cyanotoxins, which evidently play a role in competition. Apart from their independent existence, many cyanobacteria have entered into symbiotic relationships with other organisms (Adams, 2000; Rai, Söderbäck and Bergman, 2000; Rai, Bergman and Rasmussen, 2002; Bergman, Rasmussen and Rai, 2007). This phenomenon (endosymbiosis) is supposed to have initiated the complex development of the whole plant kingdom (Criscuolo and Gribaldo, 2011).

Cyanobacteria lack sexual reproduction, and multiply by cell division with various modifications of binary or multiple fission. They are the only bacterial group with well-developed clades of multicellular organisms with specialized cells and morphologically and functionally specialized parts of the thallus. Moreover, they are also the only bacteria with a plant-like metabolism, with many species capable of fixing atmospheric nitrogen; they are rightly described as carbon and nitrogen autotrophs (e.g., Stewart, 1972, 1980; Haselkorn, 1978, 1986; Wolk, 1982; Herrero and Flores, 2008). This unique ability is particularly important for survival in oligotrophic environments, as well as in many barren, human-managed, or cultured ecosystems.

Taxonomic classification is crucial for evaluating and reviewing the diversity and diversification strategies of any group of organisms. The criteria for taxonomic categorization of cyanobacteria changed with the development of other scientific disciplines. The introduction of modern methods demands more exact criteria and often a more complicated approach. During the last several years, use of electron-microscopic observations and molecular genetic criteria have greatly influenced our thinking on the diversification strategies of cyanobacteria and their taxonomic evaluation. These criteria, resulting from modern research, must therefore lead to the relevant modern classification.

2.2 Diversification and diversity

The basic structure of cyanobacteria developed more than 2.5 billions of years of years ago and there have evidently been no substantial changes in their morphology, basic physiology, and ecology up to the present. This is like having variations on a single theme (the cyanobacterial genome) for the whole period of their existence (static evolution, according to Schopf in Pennisi, 1994; Knoll and Golubić, 1992; Schopf, 1993, 1996; Castenholz, 2001). This theme is extremely successful and cyanobacteria are able to operate in all environments of the planet. Swingley, Blankenship, and Raymond (2008) made many insights into cyanobacterial evolution through comparative genomics. However, this study covers only a small part of the complex cyanobacterial diversity.

Mutation is considered the main factor in the separation of various morphological and ecological entities among cyanobacteria. This process combined with the exchange of genetic material within populations and between related populations (Rudi, Skulberg, and Jakobsen, 1998, Rudi *et al*., 2000, Rudi, Fossheim, and Jakobsen 2002; Rudi and Jakobsen, 1998; Barker *et al*., 1999, 2000; Flores and Herrero, 2005; Six *et al*., 2007) enhanced the possibilities for rapid acclimation and adaptation to a wide range of environmental conditions. Metabolic processes became readjusted and/or new proteins were induced in order to cope with new conditions (Erdmann and Hagemann, 2001; Hagemann *et al*., 2001; Hagemann, 2002; Komárek and Kaštovský, 2003). The resulting successive changes in the genomes of various taxa are sometimes rapid and may be observed during laboratory experiments.

This continuous process resulted in a large number of morphological, ecological, and ecophysiological deviations among natural populations. In fact, it is almost impossible to find two identical cyanobacterial populations or strains (Rippka *et al*., 1979; Waterbury *et al*., 1979). Some ecotypes can be stable and behave conservatively for a long time under specialized (including extreme) conditions. Other populations quickly change their properties (morphological and biochemical) after transfer into culture. This explains the plastic diversity and complicated diversification processes of cyanobacteria. However, it also complicates our approaches to systematic review of these

processes. As a result, we have to review an enormous set of morphological, ecological, and genetic modifications and deviations.

The phylogenetic relationships of cyanobacteria based on analyses of DNA sequences (sequence homology) have been accepted as the decisive criteria for their classification. This revision of cyanobacterial taxonomy was initiated in 1970s (Stanier *et al.*, 1971; Stanier and Cohen-Bazire, 1977; Tandeau de Marsac and Cohen-Bazire, 1977; Rippka, Waterbury, and Stanier, 1981). However, the first account of the phylogenetic relationships of the various cyanobacterial groups, genera, and species were published only during the last decades of the 20th century. Many workers such as Tomioka, Shinozaki, and Sugiura (1981), Giovannoni *et al.* (1988, 1990), Turner *et al.* (1989), Wilmotte and Golubić (1991), Bryant (1994), Turner (1997), Garcia-Pichel, Nübel, and Muyzer (1998), Castenholz (2001), and Litvaitis (2002) have influenced this revision of the cyanobacterial systematics (Figure 2.1).

Systematic analyses of cyanobacteria using molecular inputs often disagree with the traditional system based on morphological and, in to a small extent, ecological characteristics. Particularly morphologically simple cyanobacteria (both unicellular and filamentous) were often intermixed. This resulted in a systematic, sometimes distinctly different review from that used in classical taxonomic works. For example, using molecular genetic insights, Castenholz (2001) subdivided the cyanobacteria into a few main subsections. In this classification botanical nomenclatural rules were ignored, while taxa were selected at the traditional genus level. He used this traditional nomenclature while ignoring the rules on which they were based, without a proper revision of the systematics. The strains belonging to a genus were described, but reference strains were selected without respecting the nomenclatural rules related to typification. Further classification of sections and subsections of cyanobacteria were based on morphology (unicellular, filamentous). This approach is now considered irrelevant (cf., Schirrmeister, Antonelli, and Bagheri, 2011). However, Castenholz's use of inputs from DNA sequence analysis, alongside morphology, ultrastructure, physiology, biochemistry, and ecology, has enhanced substantially a complex revision of the cyanobacterial taxonomic system ("Guidelines for characterization of the cyanobacteria"; Castenholz, 2001), which is now commonly referred to as the "polyphasic approach" (cf. Johanssen and Casamatta, 2005; Komárek, 2006).

The modern study of cyanobacterial diversity is a complex evaluation of cyanobacterial populations and strains, in which the phylogenetic background derived from DNA sequence analysis (which is indispensable for the taxonomic classification) is integrated with the autapomorphic and ecological features of the revised taxa. The phylogenetic relationships are therefore the basis of the systematic evaluation, but other characteristics, such as cytomorphological features and ecological aspects, should also be taken into account as an integral part of the characterization of cyanobacterial taxa. The discovery and detection of correlations and coincidences between the phylogeny of cyanobacteria and their morphology and ecology represent the most exciting avenues of modern cyanobacterial taxonomy. However, in addition, the nomenclatural rules should not be ignored.

The aim of this chapter is not to describe the methods involved. In the near future, whole-genome analysis will hopefully give a more comprehensive picture of the diversity, systematics and phylogeny of cyanobacteria. The 16S rRNA gene sequences are more or less congruent with the diacritical features of the traditionally recognized genera, and are therefore an important introductory criterion for generic delimitation of cyanobacteria. The number of operational taxonomic units (OTUs) derived from the analyses of the 16S rRNA gene sequences is higher than that suggested by the classical taxonomic system based solely on morphological characters. The concept of "large and variable genera" is evidently wrong and the system must be revised by taking into account the 16S rRNA gene sequences. The use of traditional names is acceptable, but because many genera are now revised and subdivided into several new genera, the original genus name must be saved only for the one that contains the original type species.

However, the differences between clades as recognized by molecular phylogenetic analyses are still not clear. Wayne *et al.* (1987), Stackebrandt and Goebel (1994), and Stackebrandt and Ebers (2006) proposed the cut-off of 95% similarity as the criterion for the separation of taxa at the genus level. However, this artificial and sharp cut-off has been the subject of controversy and can hardly be accepted as a unique and decisive generic marker (see Ferris *et al.*, 2003;

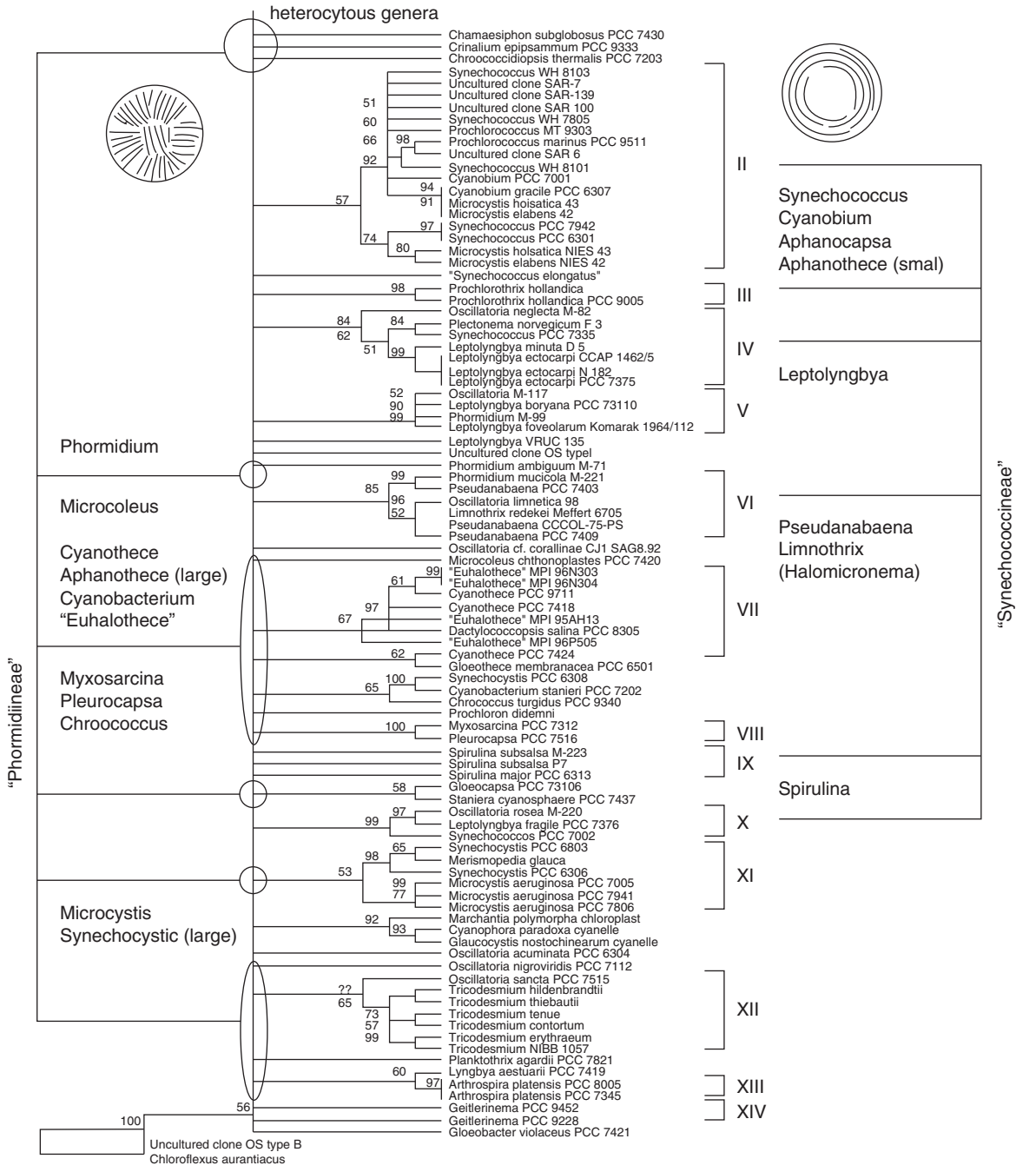

Figure 2.1 Modified phylogenetic tree of cyanobacteria from Castenholz (2001). From the tree follows the heterogeneity of coccoid and simple filamentous genera and the monophyletic origin of heterocytous genera (modified from Castenholz 2001 and Komárek 2006).

Johansen and Casamatta, 2005; and Ward, 2006 for details). Therefore, other markers are required in finalizing the classification of cyanobacteria.

The ecology of cyanobacteria is of particular importance among the taxonomically important inputs. Ecologically specific cyanobacteria exist in hypersaline environments (Garcia-Pichel, Nübel, and Muyzer, 1998), polar regions (Jungblut *et al.*, 2005, Jungblut, Lovejoy, and Vincent, 2010; Taton *et al.*, 2006; Komárek, 1999), various tropical habitats (Richert *et al.*, 2006; Turicchia *et al.*, 2009), hot springs (Kaštovský and Johansen, 2008), hot and cold deserts (Friedmann, 1980; Bahl *et al.*, 2011), and also, as picoplankton, in the oceans and in lakes (Waterbury *et al.*, 1979; Six *et al.*, 2007). Nevertheless, there are no ubiquitous cyanobacteria that can survive and form populations in all the different habitats, requiring different ecological strategies for growth (Hagemann, 2002; Garcia-Pichel, 2008). There are genotypes that are cosmopolitan and global in distribution, but these only occur under similar environmental conditions. Cosmopolitan cyanobacteria common to particular biotopes are, for example, *Microcystis aeruginosa* (plankton of eutrophic water reservoirs), *Phormidium=Microcoleus autumnale* (soils), and *Gloeocapsa sanguinea* (aerophytic wet rocks), and others that can be found only in special habitats. For example, *Mastigocladus laminosus* is a globally distributed cyanobacterium but occurs only in a special type of hot springs, within certain limits of temperature. *Geitleria calcarea* is known only from limestone caves. However, the whole issue of cosmopolitan and endemic distribution of microbes is highly speculative and subject to intense debate.

The issue of the distribution of the various infrageneric genotypes (species) of cyanobacteria is therefore closely connected with their ecology. The majority of cyanobacteria are not cosmopolitan and their distribution depends on the characteristics of the ecosystem in which they thrive. Studies have shown that some genotypes are widely distributed (e.g., *Microcystis aeruginosa*, van Gremberghe *et al.*, 2011), but other cyanobacteria such as *Phormidium retzii* (Casamatta, Vis, and Sheath, 2003) and *Cylindrospermopsis raciborskii* (Gugger *et al.*, 2005), found in widely separated areas, show a large degree of genetic diversification (Figure 2.2). The ecology and distribution of cyanobacteria must therefore be investigated in relation to the environment and geographical location in which they are found. Similar morphotypes occurring in similar, but geographically distant ecosystems very rarely belong to the same genotype, for example subaerophytic cyanobacterial communities from wet rocks (*Gloeocapsa* populations) or from the Arctic and Antarctic regions (*Phormidium*-populations; Comte *et al.*, 2007; Strunecký, Elster, and Komárek, 2010).

The "species" concept, which is commonly used in biology as the basic taxonomic unit, is now problematic for cyanobacteria in respect to modern investigations. The different concepts used to delineate "species" are untenable and problematic for cyanobacteria as well as for any asexual organisms. The definition of a recognizable species is only a conventional one. Accordingly, species is "the smallest aggregation of asexual lineages that can be diagnosed by a unique combination of characters in comparable individuals" (the phylogenetic species concept according to Nixon and Wheeler, 1990), or "group of populations and strains, which belong to one and the same genotype (genus), characterized by a stabile morphology (with distinct limits of variation), the same ecology, and occurring repetitively in similar habitats" (Komárek, 2010a, b). The proposed cut-off of 97.5% genetic similarity (Wayne *et al.*, 1987; Stackebrandt and Goebel, 1994) is highly debatable and not acceptable to all.

This situation is complicated by incorrect identifications, lack of information on morphological plasticity, and often by a complete ignorance of the ecology involved. Many authors, particularly those working with cultures, avoid the category "species" and use only strains, often designating names more or less arbitrarily. This is confusing and not acceptable for taxonomic classification. Almost all genera that are confirmed by molecular genetic methods exhibit intrageneric diversity and the classification of such units is necessary for identification practice. The category beyond the genus is especially important for ecological investigations. There are various stable populations (species) that occur in specific environments and also possess

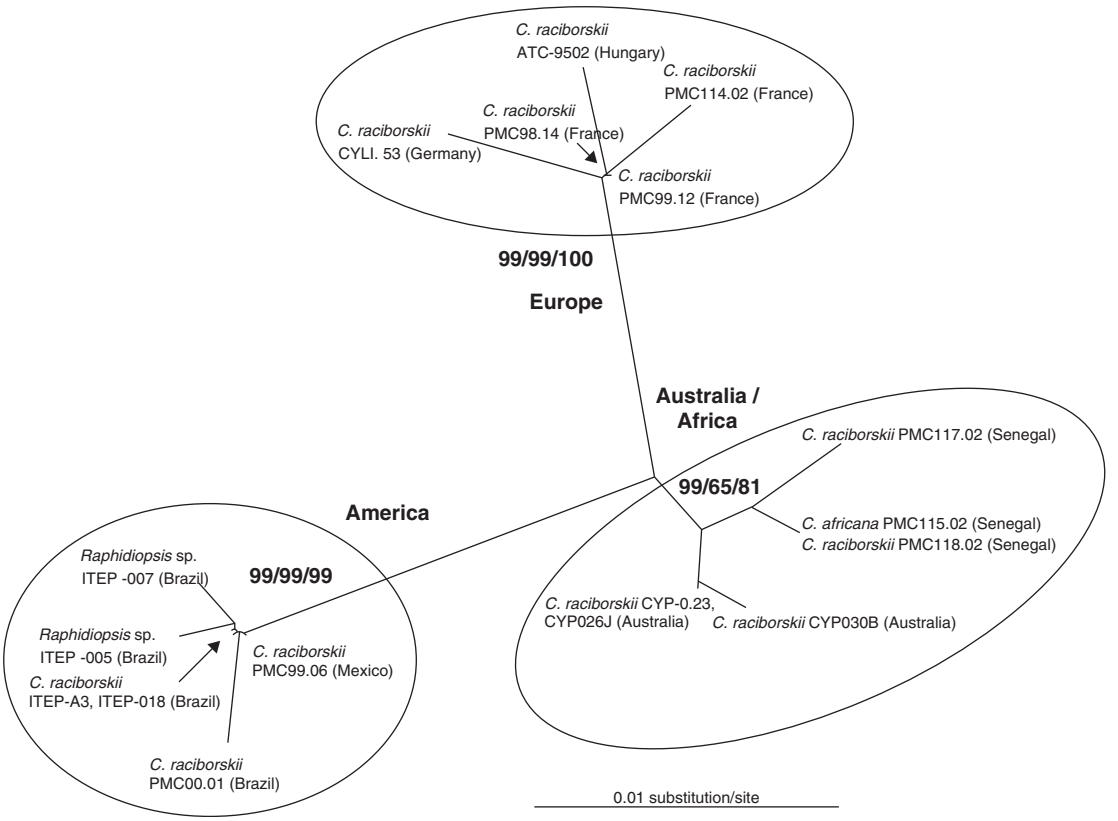

Figure 2.2 Genetic diversity of *Cylindrospermopsis raciborskii* and *Raphidiopsis* sp. populations (strains) isolated from different continents. Maximum-likelihood unrooted tree based on ITS1-L sequences (slightly adapted from Gugger *et al.* 2005, Fig. 2, with permission of the American Society for Microbiology. Copyright © 2005 by the American Society for Microbiology).

specific ecological characteristics. The determination of such units is inevitable, but the criteria for their delimitation (species delimitation) have not been (and cannot be) standardized. In nature, there exists a huge number of populations that could be new species but are not described as such.

There are also no objective criteria for the categorization of subspecific taxonomic units (varieties, forms) that could be usable for the modern classification of cyanobacteria. There is a specific category of "cryptospecies" (Sáez and Lozano, 2005; Johansen and Casamatta, 2005), which are recognized and defined by molecular criteria but are not distinguishable by any cytomorphological features. Cryptospecies should be recognized in cyanobacterial taxonomy, but there is currently no procedure to designate and register them.

2.3 The polyphasic approach

The evaluation of phylogenetic relationships based on gene sequencing is indispensable for the revision of the modern cyanobacterial taxonomic system. The sequence data of the 16S rRNA gene is most frequently used for defining generic units, and is often combined with the sequence data of the ribosomal internally transcribed spacer (ITS) or other genes in order to achieve a higher taxonomic resolution. The concatenation of a set of gene sequences is often used for a more solid phylogenetic analysis. However, other characters (cytomorphological, ecophysiological, biochemical) must not be omitted. The discovery of

coincidences and correlations between the phylogeny and other characteristics is an important task of the cyanobacterial taxonomist and should be applied to all future revisions (Castenholz, 2001; Johansen and Casamatta, 2005; Komárek, 2006).

Additional methodological principles are as follows:

1. The morphological characters of various genotypes must be defined and included. The correct descriptions of autapomorphic characters are preferred, and should constitute an integral part of the definition of taxa. An example of phylogenetic clades characterized by autapomorphic characters is the revised review of oscillatorialean genera (Figure 2.3). Characters considered as variable are sometimes in agreement with genetic analyses (e.g., in terms of morphology, the length of cells, width of trichomes, morphology of terminal cells, morphology and position of akinetes, presence of gas vesicles in cells, and others) and the limits of the variability of various markers must be recognized. Morphological studies are important also for the possibility to utilize the whole body of knowledge about cyanobacteria built up over the 150-year period of their investigation. For cryptospecies, information about autapomorphic characteristics is missing, and their evaluation remains unresolved in the cyanobacteria taxonomic classification.

2. Ultrastructure is important. The relationship of several specialized cell structures with the phylogenetic position of the organism is poorly understood (e.g., widened thylakoids, spirally coiled thylakoids, various crystals and organic features, and the facultative occurrence of gas vesicles). For example, the position of thylakoids is congruent with several cyanobacterial clades (Figure 2.4). However, the various modifications of the position and morphology of the thylakoids are not fully elucidated and some variability (e.g., in response to the light intensity) exists for this marker.

3. Ecological and ecophysiological specificity is an important marker for the characterization of various taxa. Adaptation to extreme environments plays an important role in diversification in cyanobacteria. Examples include the clade of the thermophilic *Synechococcus* (*Thermosynechococcus*; Katoh *et al*., 2001), tropical genera such as *Brasilonema* and *Streptostemon* (Fiore *et al*., 2007, Sant'Anna *et al*., 2011), unicellular halophilic cyanobacteria (*Halothece* and *Euhalothece*; Garcia-Pichel, Nübel, and Muyzer, 1998) and filamentous halophiles (*Oxynema;* Chatchawan *et al*., 2012).

4. Ecophysiological and biochemical characters are important for the characterization of some cyanobacterial taxa. However, they have been little studied.

The majority of experimental studies are conducted on laboratory strains of cyanobacteria. These data need to be verified by results obtained from natural samples. During culturing, natural populations that are adapted to specific local conditions are transferred to more-or-less standardized laboratory conditions, which differ considerably from the original habitat. Cultivation under standard conditions may quickly result in a drastic change in the physiology and morphology of the organisms. The genetic background of these changes in most cases remains unknown. The behavior of cultured strains may differ between genera and species. Some types with a distinct ecology have not been cultured (*Gloeocapsa*, *Hyella*, numerous species of *Stigonema*, and others). This failure is evidently connected with the specific ecological demands of these ecotypes. The use of strains is of course inevitable in cyanobacterial taxonomy but the issues mentioned above need to be respected.

The evaluation of taxa resulting from the polyphasic approach is sometimes difficult. There are different strategies for the infrageneric differentiation of the cyanobacterial genera as they are recognized from sequence analysis. These approaches often show a high diversity and result in the splitting up of taxa but the new delimitations are difficult to categorize (differently in different genera).

Another problem in modern cyanobacterial taxonomy is the nomenclature. Stanier *et al*. (1978) proposed the transfer of cyanobacteria from the legislation of the International Committee of Botanical Nomenclature (ICBN) to that of the International Code of Nomenclature of Prokaryotes (ICNP). However, this proposal appeared to be complicated and raised numerous new questions. The biggest problem was that neither code was entirely acceptable for cyanobacterial taxonomy (e.g., in the problems of typification). Moreover, several newly isolated model genera and strains are insufficiently described and not valid according to either of the two codes (e.g., "*Cyanothece*" sensu Rippka, *Crocosphaera*, *Thermosynechococcus*, and oceanic "*Synechococcus*").

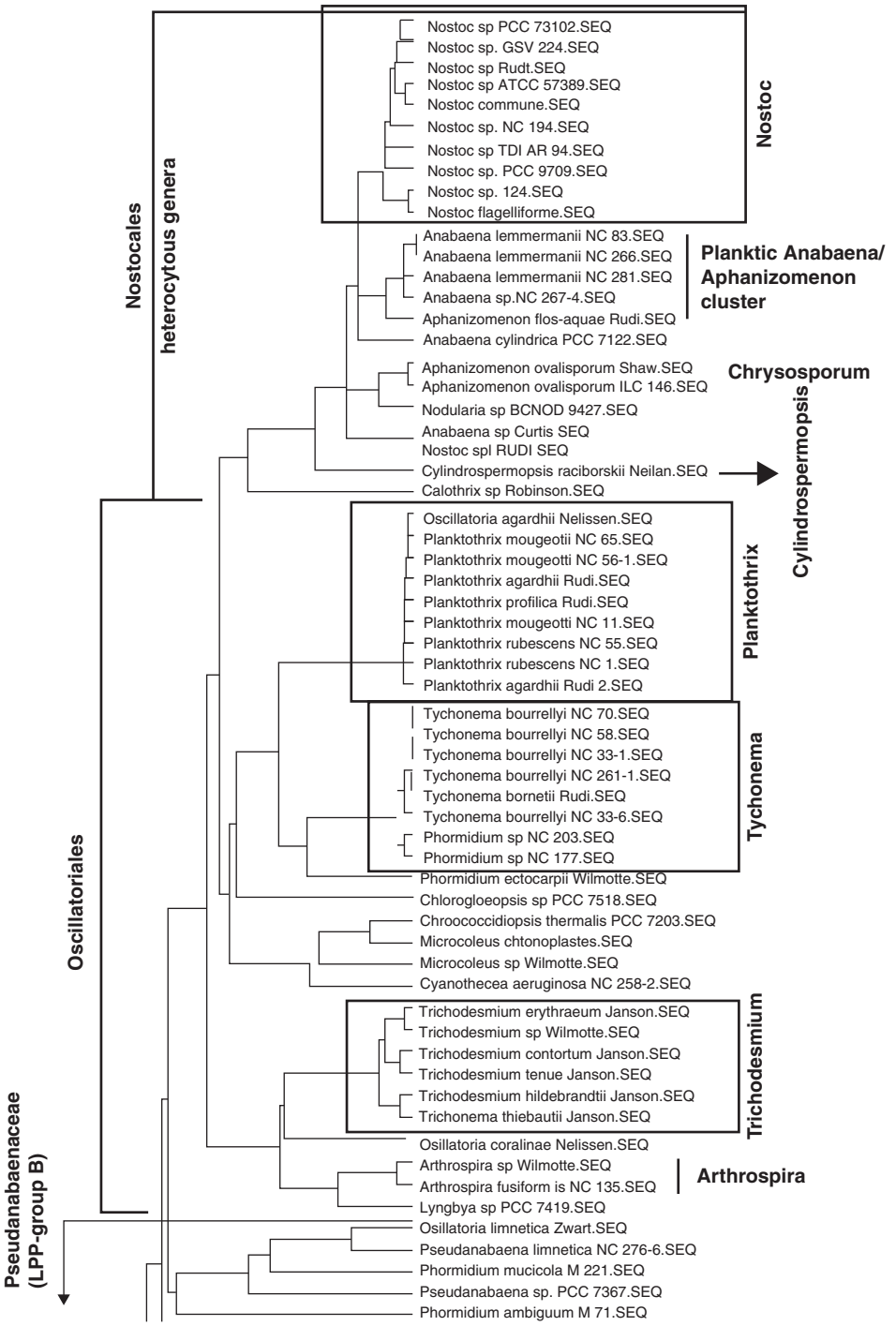

Figure 2.3 Phylogenetic integrity of morphologically defined genera from trichal cyanobacteria according to Komárek and Anagnostidis (2005) (Komárek 2006. Reproduced with permission of The Korean Society of Phycology).

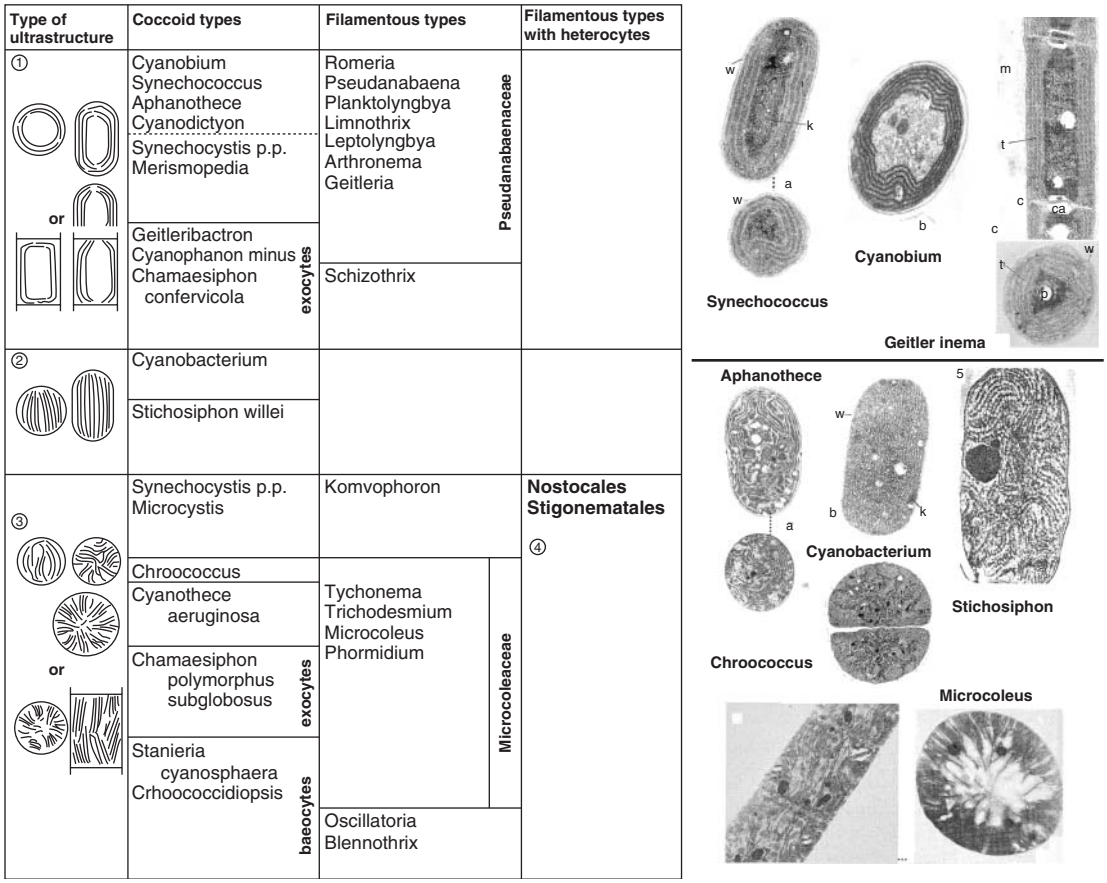

Figure 2.4 Example of characteristic position of thylakoids in cells in clusters, evaluated by combined molecular and morphological markers (Komárek 2006. Reproduced with permission of The Korean Society of Phycology).

The binominal nomenclature is widely used in cyanobacterial research, but it is connected to the need of typification according to the ICBN. The complications of cyanobacterial morphological and ecological specificity cause many problems for the application of bacteriological nomenclatural rules. Attempts have been made to solve this problem (Oren, 2004, 2011; Oren and Tindal, 2005; Oren and Komárek, 2010), but still without any success. The ICNP accepts all cyanobacterial taxa that have been defined according to the botanical rules, but this is a provisory recommendation. Komárek and Golubić (2005) have combined the bacteriological and botanical rules and proposed a nomenclature for cyanobacteria (see www.CyanoDB.cz.). However, their proposal has not yet been accepted and currently either mixtures of rules of both codes are applied, or a traditional description of taxa according to the Botanical Code is followed.

The use of the binomial nomenclature is particularly important for experimental work and the application of molecular methods in cyanobacterial taxonomy. It is confusing to use the results of sequencing for taxonomic classification when only the strain designations are used (sometimes very arbitrarily). In particular, the rules for typification (as proposed by ICBN) must be accepted as well as the rules for the identification of "reference" strains. The complication is that both of these indispensable and essential requirements are not tuned to each other, resulting in arbitrarily

selected strains being designated reference strains without reference to the original types. However, progress in cyanobacterial taxonomy is possible only when the significant rules of both codes are respected.

2.4 Selected changes in the modern system

When new results become available, the taxonomic system for cyanobacteria must be revised continually as a product of molecular (phylogenetic) analysis combined with phenotypic and ecophysiological characteristics. A few recent examples of important changes in cyanobacterial classification are presented here; these have emerged from the revisions of the cyanobacterial taxonomic system.

1. The traditional rectilinear system is based on the scheme: unicellular genera → (unicellular baeocytic and exocytic types) → undifferentiated filamentous genera → heterocytous (unbranched, false, and true branched) types. This does not agree with the phylogeny based on the sequence of the 16S rRNA gene (cf., Castenholz, 2001; Komárek and Kaštovský, 2003; Schirrmeister, Antonelli and Bagheri, 2011). Therefore, modified systematic schemes have been developed that respect the phylogeny and the ultrastructure (Hoffmann, Komárek and Kaštovský, 2005a, 2005b; Komárek and Anagnostidis, 2005; Komárek and Kaštovský, 2013). The revision of cyanobacterial systematics is still in its infancy, but it already shows the consequences in terms of the relationships between different genera and groups of cyanobacteria, and provides guidelines for further research. It is important to discover the correlations and coincidences of phylogenetic and phenotypic markers of different taxa, as for instance the difference in ultrastructure of the thylakoids in phylogenetically unrelated cyanobacteria. The revised systematics of cyanobacteria are depicted in Figures 2.5 and 2.6.

2. Unicellular and undifferentiated filamentous cyanobacteria are particularly heterogeneous. Hence the traditional Chroococcales must be divided into several clades. The unicellular types with parietal thylakoids (*Anathece*, *Cyanobium*, *Cyanodictyon*, *Synechococcus*, *Thermosynechococcus*) are closely related to the tiny filamentous Pseudanabaenaceae (*Arthronema*, *Geitlerinema*, *Leptolyngbya*, *Planktolyngbya*, *Pseudanabaena*, *Romeria*), while the distinctly distant group of Chroococcaceae, with a more complicated intracellular structure (see *Chroococcus* like types in Figure 2.4), is more related to the bigger filamentous Oscillatoriaceae or Nostocaceae. Transient forms with specific modifications in the ultrastructure occur in some groups.

3. Most traditional genera of filamentous, non-heterocytous groups are heterogeneous (*Geitlerinema*, *Leptolyngbya*, *Phormidium*, *Microcoleus*, *Oscillatoria*, *Lyngbya*). They are being revised as new knowledge becomes available and numerous new genera have been proposed (Boyer *et al.*, 2002; Siegesmund *et al.*, 2008; Abed, Garcia-Pichel and Harnandez, 2002; Strunecký, Elster, and Komárek, 2011; Engene *et al.*, 2012). The new genera *Annamia, Halomicronema*, *Nodosilinea*, *Oculatella*, *Planktolyngbya* and *Plectolyngbya* were separated from the Pseudanabaenaceae (especially from *Leptolyngbya*). *Phormidesmis* and *Wilmottia* were separated from the traditional genus *Phormidium*. The new genera *Aerosacconema*, *Coleofasciculus*, *Limnoraphis*, *Moorea*, *Oxynema*, and *Roseofilum* were described in the oscillatorialean group. Numerous other groups still need to be investigated for their proper taxonomic placement. Moreover, traditional genera are being revised, confirmed, and delimited according to the type species and related taxa (e.g., *Microcoleus*, *Lyngbya*, *Phormidium*, and *Planktothrix*).

4. Some cyanobacteria with an unusual structure of the photosynthetic system (absence of thylakoids, presence of a typical photosynthetic pigments such as chlorophyll *b* and *d*) have been discovered and their phylogenetic relationship to other cyanobacteria has been established (*Gloeobacter*, *Prochloron*, *Prochlorococcus*, *Prochlorothrix*, *Acaryochloris*). Such unusual cyanobacteria are important for developing an understanding of the evolution of the whole group, but their taxonomic position is not yet fully determined.

5. The presence or absence of genes for the formation of gas vesicles is a stable marker and can be used for taxonomy. The formation of aerotopes (clusters of gas vesicles) is characteristic of several genera of unicellular as well as oscillatorialean and heterocytous cyanobacteria. They include the unicellular *Microcystis*, the

filamentous genera *Planktothrix*, *Trichodesmium*, and *Limnoraphis*, and the heterocytous genera *Anabaenopsis*, *Aphanizomenon*, *Cuspidothrix*, *Cylindrospermopsis*, and *Dolichospermum*. Other groups do not form these clusters of gas vesicles (e.g., the oscillatorialean type *Arthrospira* and the heterocytous genus *Nodularia*). In *Hapalosiphon*, clusters of gas vesicles are only formed in hormogonia. Other important properties such as nitrogen fixation, the precipitation of calcium carbonate in sheaths, and the production of cyanotoxins are found throughout the cyanobacterial domain and are not confined to specific phylogenetic groups. The production of specific cyanotoxins is found in several genera that are sometimes not closely related (e.g., cylindrospermopsin, nodularin). The explanation of this wide distribution of toxins is a challenging question in cyanobacterial diversification.

6. A special position was recognized for a group of unicellular baeocytic cyanobacteria. They possess a complicated and diversified thallus, reproduction strategy, and cell structure. Many of these cyanobacteria live in extreme environments (thermal springs, endolithic habitats, hypersaline environments) and they are difficult to cultivate. These cyanobacteria occupy a well-defined position in the phylogenetic tree of cyanobacteria and are separated from other unicellular forms. Studies addressing the phylogenetic position are expected in the more diversified types (such as *Cyanoderma*, *Cyanosaccus*, *Dalmatella*, *Hyella*, and *Pleurocapsa*).

7. The heterocytous cyanobacteria represent a monophyletic clade. However, they contain several distinct lines, distinguishable according to phylogenetic and morphological criteria. They were classified as the orders Nostocales (without branching of the trichomes or with false branching, when two trichomes come together within one sheath) and Stigonematales (characterized by true branching, after cell division in more than one plane). However, various types of branching (both false and true) are characteristic only for a few separated clusters and they cannot be used for primary classification inside the heterocytous types (Gugger and Hoffmann, 2004). The taxonomic value of branching is limited to some families (e.g., to Scytonemataceae, Symphyonemataceae, Stigonemataceae, Fischerellaceae, and Hapalosiphonaceae).

8. Many traditional families are heterogeneous and should be subdivided or re-evaluated. The new family Symphyonemataceae is characterized by a distinct position in the phylogenetic tree and its possessing trichomes with true reverse Y-type branching (Gugger and Hoffmann, 2004). In the family Rivulariaceae, several groups of the genus *Calothrix* (with akinetes, without typical hairs) should be separated and the genus *Gloeotrichia* that can form akinetes should be separated from the rivulariacean genera without akinetes. The family Microchaetaceae, characterized by heteropolar trichomes and the absence of terminal hairs, comprises several phylogenetically distant clusters and should be divided into different taxonomic groups.

9. Akinetes are characteristic of the Nostocaceae and a few genera belonging to other families. The latter genera seem to be closely related to the nostocacean group (*Gloeotrichia* or akinete-forming *Calothrix*). The dormant cells (arthrospores) that are found in other families (for instance in Scytonemataceae and Stigonemataceae) have a structure and morphology different from akinetes.

10. The majority of traditional heterocytous genera are heterogeneous and should be subdivided based on sequence analysis of the 16S rRNA gene. There are numerous short reviews of newly defined genera from heterocytous cyanobacteria (Flechtner *et al.*, 2002; Rajaniemi *et al.*, 2005; Willame *et al.*, 2006; Sihvonen *et al.*, 2007; Komárek, 2010a, b; Stűken *et al.*, 2010; Zapomělová *et al.*, 2009, 2010; Berrendero, Perona and Mateo, 2011; Komárek and Mareš, 2012); see also Table 2.1.

11. Ecological and phytogeographical data are important for the definition and revision of all cyanobacterial taxa.

12. The terminology of cyanobacteria has been corrected and revised using improved knowledge of the function of subcellular structures. Scientific terminology needs improvement and should be revised in accordance with the progress in science. The acceptance of new terminology is slow but its use is highly recommended. Examples include the use of the term baeocytes instead of endospores, exocytes instead of exospores, aerotopes instead of gas vacuoles, and heterocytes instead of heterocysts.

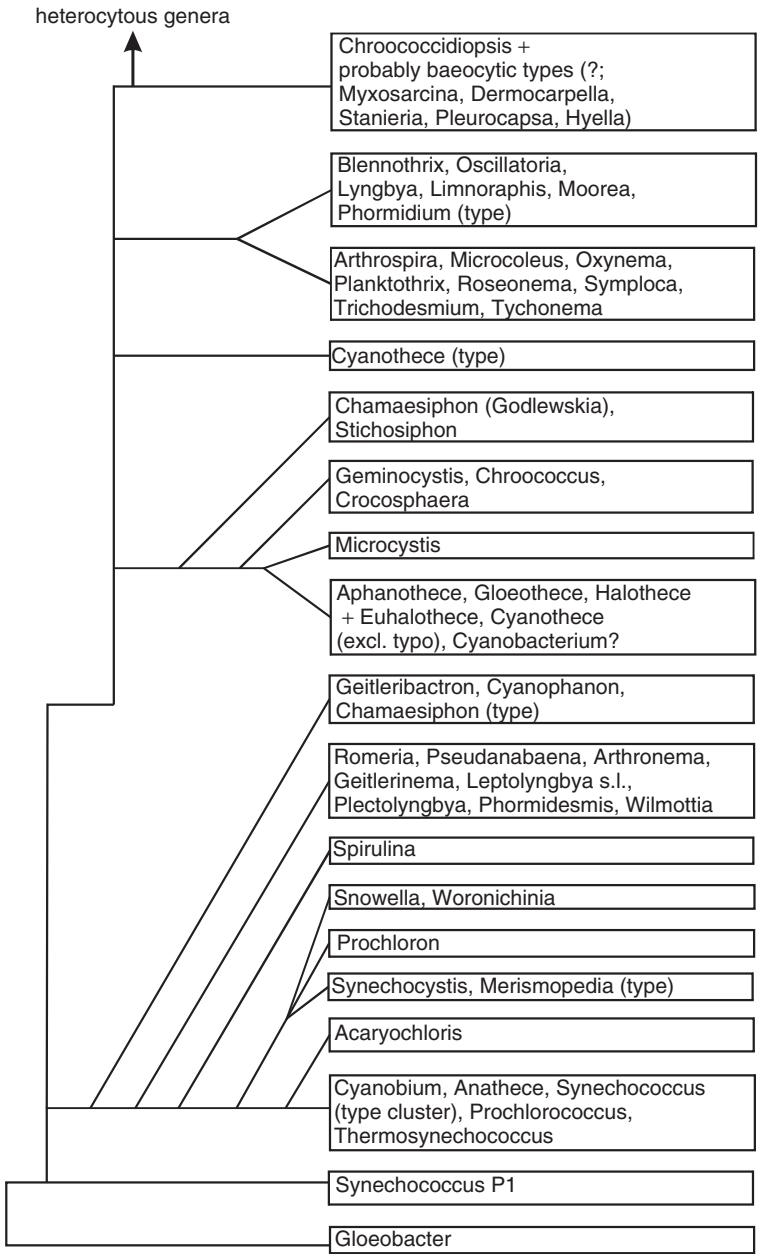

Figure 2.5 Scheme of the present taxonomic classification of coccoid and filamentous cyanobacteria without heterocytes using modern polyphasic approach, especially 16S rRNA gene sequencing. Only the main selected genera are included. The clusters can be classified as respective families. The lines express the relationships of the various groups (estimated from various criteria of polyphasic evaluation).

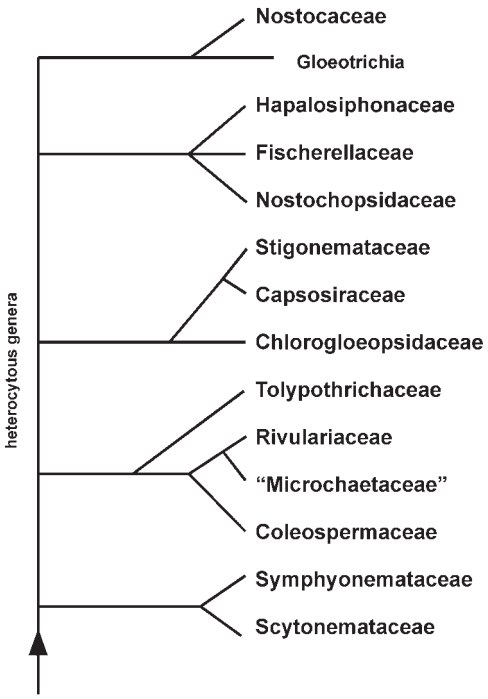

Figure 2.6 Scheme of the present taxonomic classification of families of heterocytous cyanobacteria according to the modern polyphasic approach. (The names of families are provisional and invalid and correspond only to the present state of the cyanobacterial system taking into account the main changes resulting from modern revisions).

Table 2.1 Examples of traditional cyanobacterial genera divided in various genera according to modern polyphasic approach. Several modern defined genera belong in different phylogenetic clades.

Traditional genera according to morphological taxonomy	Genera defined according to modern polyphasic approach
Synechococcus	*Cyanobacterium, Cyanobium, Cyanothece, Halothece, Prochlorococcus, Synechococcus, Thermosynechococcus*
Synechocystis	*Geminocystis, Synechocystis*
Aphanothece, Gloeothece	*Anathece, Aphanothece, Cyanoaggregatum, Gloeobacter, Gloeothece*
Pseudanabaena	*Annamia, Arthronema, Komvophoron, Pseudanabaena*
thin Oscillatoria, thin Phormidium, thin Lyngbya	*Halomicronema, Leptolyngbya, Limnothrix, Nodosilinea, Oculatella, Phormidesmis, Planktolyngbya, Plectolyngbya, Wilmottia*
Spirulina	*Arthrospira, Halospirulina, Spirulina*
Phormidium	*Oxynema, Phormidium, Planktothrix, Roseofilum;* one cluster transferred to *Microcoleus*
Lyngbya	*Eulyngbya, Limnoraphis, Lyngbya, Moorea*
Anabaena	*Anabaena, Chrysosporum* (part), *Cronbergia, Dolichospermum, Macrospermum, Sphaerospermopsis, Trichormus, Wollea* (revid.)
Aphanizomenon	*Aphanizomenon, Chrysosporum* (part), *Cuspidothrix*

2.5 Main prospects, importance for biotechnology

Revision of the cyanobacterial systematics taking into account the phylogeny, based particularly on sequence analysis of the 16S rRNA gene, is still in its infancy. The revision using the polyphasic approach is necessary but it requires further investigation. The cultivation of many cyanobacteria is difficult and many species that have been described from natural sources have not been taken into culture. The scientific community only slowly accepts new insights. This conservatism goes often hand in hand with a poor knowledge of the relevant literature, ignorance of the nomenclatural rules or their erroneous application, and the use of different taxonomic concepts. The designation of strains by different, alternative symbols and the arbitrary use of binomial names (in various databases and in GenBank) are particular impediments to progress in cyanobacterial classification. Therefore, it is urgent that the scientific community accepts new insights in cyanobacterial taxonomy and implements them in experimental and ecological studies in order to avoid confusion with respect to the identity of the reported strains and species.

Taxonomic classification remains the only method for recognition of the diversity of a group of organisms. The conclusions of taxonomic and diversity studies are important for all other scientific and applied disciplines. For the characterization of recognized taxonomic units (OTUs), phylogenetic relationships are important in addition to morphological variability, life cycles, and ecophysiological and biochemical properties and function in ecosystems. The scientific

name of the organism is a symbol of all these properties and correct identification is therefore essential. In many biological and applied disciplines corrections to the classification of organisms based on state-of-the-art knowledge is evidently required. It is remarkable that within the field of cyanobacteria study, taxonomic changes have often been ignored for many years. For instance, the name "*Anacystis nidulans*" still appears in the literature although its misidentification has been recognized for more than 40 years. The incorrectness of the name "*Spirulina platensis*" (instead of *Arthrospira*), a cyanobacterium known from its successful mass cultivation and use as a food additive, has been recognized for 30 years. Studies of "*Synechococcus*" or "*Cyanothece*" strains that do not correspond to the recognized characteristics of the types of genera *Synechococcus* and *Cyanothece* are still commonly published in many papers. Cyanobacterial strain collections, which accept the superficially identified strains and ignore the later revisions should take the blame. A large number of incorrectly identified cyanobacteria can be found in GenBank and experimental papers often use curious names, such as "unidentified cyanobacterium", "Antarctic uncultured strain", "*Cyanobacterium* sp.", "*Nostoc Anabaena*", and others. Such designations are the main source of mistakes and correct identification of strains should be demanded before a strain's use in experimental studies or its acceptance to a culture collection.

Cyanobacteria are gaining attention for their potential biotechnological applications, such as for the production of bioactive compounds, or use as fertilizers or fuels. Correct identification of strains in biotechnological activities is therefore not only a requirement for the satisfaction of taxonomists, but is the only method that can characterize the organism and help to solve the many biotechnological problems.

Future work in cyanobacterial taxonomy will evidently focus on the complex evaluation of genomes. The basis for the classification of cyanobacterial diversity will be the elucidation of the whole genome, with identification of consequent autapomorphic markers and ecological limits. Therefore in the coming years the following aspects must be taken into account:

- the consistent use of polyphasic methods, with obligatory phylogenetic analysis and studies of comparative genomics, in various cyanobacterial types
- the definition of autapomorphic markers and ecological characterization of resulting taxonomic units
- control and consistent application of evaluation of taxonomic units at various levels
- solution to the problem of cryptic species (characterized only by genetic differences)
- respecting of nomenclatural rules; this is the inevitable precondition for any successful progress in cyanobacterial taxonomy.

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PART II

Ecological services rendered by cyanobacteria

Chapter 3
Ecological importance of cyanobacteria

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3.1 Introduction

Cyanobacteria have, for billions of years, shaped the biogeochemistry of Earth like no other organisms. They introduced elemental oxygen to the atmosphere during the Proterozoic (from 2.5–544 million years ago) and became the main primary producers of organic matter on the planet. In the early evolution of life, chloroplasts of today's algae and higher plants originated from a cyanobacterial endosymbiotic relationship with an ancient eukaryotic cell (Martin and Kowallik, 1999; Raven and Allen, 2003), resulting in oxygen-tolerant organisms capable of aerobic respiration. Their ancient origin and biogeochemical importance is reflected in their colonization of a wide variety of aquatic and terrestrial habitats. They exist in cold and hot climates, being key players in global cycling of both carbon and nitrogen, and through their versatile ecophysiological strategies they can thrive in both nutrient-poor and polluted habitats. Cyanobacteria often live in association with other organisms, either symbiotically or epiphytically, or by forming microbial mats and biofilms. These associations are the predominant and sometimes the only life forms found in certain habitats, such as cold and hot deserts all over the planet.

Cyanobacteria are a morphologically and genomically diverse group. They exist as unicellular (single or agregated cells), filamentous (thin or thick, single trichome, or in beams, and with or without sheath), and branched forms (Castenholz, 2001). Up to June 2012, genomes of 202 different isolates of cyanobacteria have been or are being sequenced (www.genomesonline.org). The sequenced genomes display a range of sizes (1.6 Mb to 8–9 Mb), with genomes of the unicellular and in particular the marine *Prochlorococcus* and *Synechococcus* genera being the smallest (1.67–2.22 Mb). Moreover, a large amount of metagenomic data is being generated from environmental

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sequencing projects around the globe. A substantial resource for exploring phylogenetic relationships, metabolic pathway, and enzymes is being generated at a rapid pace. The continuous exploration of little-studied environments with high abundances of cyanobacteria is resulting in the identification of new strains, enzymes, and metabolites with both commercial and ecological importance.

3.2 Variation in cyanobacterial light-harvest pigments

The varied cyanobacterial photopigments generate a great diversity in color (Stal, 2007). Apart from chlorophyll *a* (green), the phycobiliproteins present in the phycobilisomes – phycocyanin, allophycocyanin (blueish), and phycoerythrin (reddish) – are the principal accessory pigments enabling cyanobacteria to effeciently use the light spectrum between the absorption peaks of chlorophyll *a* and the carotenoids. They exhibit several types of chromatic adaptation, regulated at transcriptional and postranscriptional levels (Palenik, 2001; Gutu and Kehoe, 2011). These adaptations result mainly from changes in the ratios of phycocyanin and phycoerythrin (Tandeau de Marsac, 1977). Furthermore, thermophilic cyanobacteria may possess different types of phycocyanins compared to the mesophilic species in order to better resist thermal denaturation (Samsonoff and MacColl, 2001).

Certain cyanobacteria (e.g., the prochlorophytes) lack phycobilisomes and phycobiliproteins and are therefore important exceptions in terms of cyanobacterial pigment composition. The marine unicellular *Prochloron didemni* and the filamentous cyanobacterium *Prochlorothrix hollandica* contain chlorophyll *b*, while the marine genus *Prochlorococcus* contains divinyl derivatives of chlorophyll *a* and *b* (Goericke and Repeta, 1992). A further exception is *Acaryochloris marina*, a unique phototroph living on coral reefs in association with didemnid ascidians (Marquardt *et al.*, 1997) and in other endolithic habitats (Behrendt *et al.*, 2011). *A. marina* uses chlorophyll *d* as their main light-harvesting pigment, enabling far-red light harvesting. A new type of chlorphyll termed "*f*" has been reported in stromatolites built by cyanobacteria (calcified, stratified microbial mats) in Shark Bay, Australia (Chen *et al.*, 2010).

The wide array of pigments and their chromatic adaptation allows cyanobacteria to occupy a range of different habitats as well as to coexist by occupying different light-related niches (Stomp *et al.*, 2004). A comparison of pigment content and the genes encoding pigments have been used to predict changes in the spatial distribution of cyanobacterial ecotypes in aquatic systems (Haverkamp *et al.*, 2008; Acinas *et al.*, 2009; Bibby, Zhang, and Chen, 2009).

3.3 Metabolic diversity in cyanobacteria

Cyanobacteria inhabit a wide range of environments. They grow in saline, brackish, and fresh water, as well as in cold environments, and hot springs and deserts, in many cases where no other microalgae can exist. They require nitrogen as an essential macronutrient, with ammonia as the favored source (Herrero, Muro-Pastor, and Flores, 2001). However, they can also assimilate nitrate and urea. Some species can even fix molecular nitrogen. Thus, they are unique in their ability to simultaneously perform oxygenic photosynthesis and oxygen-sensitive nitrogen fixation. Nitrogen-fixing cyanobacteria need, as a minimum, nitrogen, carbon dioxide, water, and some mineral elements for their growth.

Though all cyanobacteria are fundamentally oxygenic photosynthetic organisms, some species can switch their metabolic mode to, for example, anoxygenic photosynthesis, using sulfide (Cohen *et al.*, 1986; Garcia-Pichel and Castenholz, 1990) and ferrous iron (Widdel *et al.*, 1993) as electron donors. Under dark and anoxic conditions, some cyanobacteria also perform fermentation (Stal and Moezelaar, 1997 and references therein; Steunou *et al.*, 2006). In the dark, intracellularly stored carbohydrate is consumed through aerobic respiration, using oxygen as the terminal electron acceptor. Under conditions where elemental sulfur, ferric iron, or protons are present, they can optimize the energy efficiency of fermentation by using these as electron sinks, thereby producing

sulfide, ferrous iron, or hydrogen gas, respectively. The switch to fermentation can be instantaneous, as the enzymes needed are constitutively expressed (Stal and Moezelaar, 1997 and references therein). Furthermore, there are cases of mixotrophy or even heterotrophy among cyanobacteria (Fay, 1965; Eiler, 2006; Béjà and Suzuki, 2008). Some cyanobacteria have the ability to live as heterotrophs on organic compounds under both dark and light conditions. For instance, glycogen synthesized in the presence of light may act as the main storage for energy and carbon, but is then mobilized for use in the dark, either in aerobic respiration where oxygen is present or in fermentation otherwise. Photoheterotrophy and chemoheterotrophy have been observed among unicellular cyanobacteria such as *Cyanothece*, *Aphanocapsa*, *Chlorogloea*, and *Agmenellum quadruplicaturm* (Rippka, 1972; Lambert and Stevens, 1986; Feng *et al.*, 2010). Marine picocyanobaceria *Synechococcus* and *Prochlorococcus* have been suggested as being mixotrophic and putative competitors to heterotrophic bacteria for amino acids, nucleosides (Moore *et al.*, 2002; Zubkov *et al.*, 2003; Michelou, Cottrell, and Kirchman, 2007), and methylated sulfur compounds such as dimethyl-sulfoniopropionate and methanethiol (Malmstrom *et al.*, 2005). Uptake of amino acids and other organic compounds have also been evident in other cyanobacteria such as *Synechocystis*, *Pseudanabaena*, *Anabaena*, *Nostoc*, *Calothrix*, *Fischerella* and *Planktothrix rubescens* under light stimulation (Montesinos, Herrero, and Flores, 1997; Zotina, Köster, and Jüttner, 2003). Chemotaxis toward nitrogenous compounds was found in *Synechococcus* (Willey and Waterbury, 1989). Furthermore, uptake of organic sugar componds as both energy and carbon sources in darkness has been reported in *Nostoc*, *Arthrospira*, *Oscillatoria*, *Gleitlerinema*, and *Microcystis* (Stal and Moezelaar, 1997 and references therein; Mühling, Belay, and Whitton, 2005; Richardson and Ragoonath, 2008). Moreover, amino-acid-transport genes have been reported in *Anabaena*, *Nostoc*, *Pseudanabaena*, and *Planktothrix* (Montesinos, Herrero, and Flores, 1997; Zotina, Köster, and Jüttner, 2003), and urease coding genes in *Synechococcus* (Collier, Brahamsha, and Palenik, 1999; Palinska *et al.*, 2000). Rocap and co-workers (2003) found oligopeptide transporter genes in *Prochlorococcus*. Heterotrophy was reported in the unculturable unicellular free-living nitrogen-fixing cyanobacteria UCYN-A (Zehr *et al.*, 2008). This conspicuous cyanobacterial strain exhibited a unique survival strategy, without oxygen-evolving photosystem II, and involving photofermentation (Bothe, Tripp, and Zehr, 2010; Tripp *et al.*, 2010).

3.4 Intra- and extracellular products facilitating cyanobacterial survival

Cyanobacteria have the ability to assimilate and store key nutrients and metabolites for future consumption. Filamentous heterocystous cyanobacteria are differentiated by akinetes, cells containing reserve compounds that enable survival under stress or less optimal environmental conditions. In addition, cytoplasmic inclusions such as glycogen granules, lipid globules, cyanophycin granules, polyphosphate bodies, and carboxysomes have also been observed (Fay and Van Baalen, 1987). Many of these compounds have relevant industrial applications. For example, polyhydroxyalkanoates generated under excess carbon but nitrogen-limiting conditions could be used as bioplastics (Stal, 1992 and references therein), or as biocompatible material in biomedicine and biopharmaceutics (Sudesh, 2004). Cyanophycin (non-ribosomal polypeptide), a nitrogen-storage body (Simon, 1971), also acts as a potential energy storage material (Smith, 1982). Phycobiliproteins that are degraded under nitrogen (or sulfur) limitation have been suggested as the basis of a nitrogen storage system (Wyman, Gregory, and Carr, 1985; Kana, Feimel, and Flynn, 1992). In addition, many cyanobacteria accumulate phosphate as polyphosphates (Healey, 1982), which can be used as pesticides, detergent additives, and antibiotics (Nowack, 2003; Singh, 2009 and references therein).

Many cyanobacteria produce extracellular polysaccharide (EPS) sheaths, which act as a buffer zone between the cell and the environment, and contribute to desiccation tolerance. Desiccation resistance is further aided by the production of sheath pigments such as gloeocapsin, fuscorhodin, and fuscochlorin (Bewley, 1979 and referentes therein; Helm *et al.*, 2000).

EPS composition and structure vary among cyanobacterial taxa, and also within the same species at different stages of growth or depending on the environment (Stal and Krumbein, 1985). Cyanobacterial EPS has been applied in the textile industry to remove dyes from effluent and for oil recovery, as it increases oil solubility and mobility (Abed, 2009).

In cyanobacteria, quorum sensing (gene expression regulation using chemical signal molecules) has been shown in response to cell-population density fluctuations, and this might enable cooperative behavior to increase their fitness. Studies have demonstrated the production of the quorum-sensing acylhomoserine lactones, affecting biofilm formation (Sharif *et al.*, 2008) and nitrogen fixation (Romero, Muro-Pastor, and Otero, 2011). Quorum-sensing inhibitory activities under natural conditions have been observed in cyanobacteria inhabiting microbial mats in extreme hypersaline systems (Abed *et al.*, 2011; Dobretsov, Abed, and Al Maskari, 2011).

Cyanobacteria show allelopathic interactions with other organisms in the community, releasing biochemical compounds (allelochemicals) to the surrounding environment. Furthermore, they produce antibacterial (Jaki, Heilmann, and Sticher, 2000; Biondi *et al.*, 2008, Dobretsov, Abed, and Al Maskari, 2011), antifungal (Biondi *et al.*, 2008), antiviral (Patterson, Larsen, and Moore, 1994; Rajeev and Xu, 2004), algicidal (Dahms, Xu, and Pfeiffer, 2006 and references therein; Dobretsov, Abed, and Al Maskari, 2011), and immunosuppressive agents (Koehn, Longley, and Reed, 1992). Some cyanobacteria (e.g., *Lyngbya majuscula*; Burja *et al.*, 2001; Shimizu, 2003) are known to produce bioactive compounds that exhibit a wide spectrum of biological activities. The most important type of compounds produced by cyanobacteria to help them compete in their environment are those targeting other cyanobacteria and microalgae, and these can also be used to control algal blooms (Dahms, Xu, and Pfeiffer, 2006). Several of these allelochemicals have been identified. For example, cyanobacterin is produced by *Scytonema hofmanni* (Mason *et al.*, 1982), fischerellin A by *Fischerella muscicula* (Hagmann and Juttner, 1996), hapalindoles by *Hapalosiphon* (Moore, Cheuk, and Patterson, 1984) and *Fischerella* spp. (Doan *et al.*, 2000), and nostocyclamides by *Nostoc* sp. (Juttner *et al.*, 2001). In addition, cyanotoxins have also been proposed as allelochemicals: molecules that are involved in inter-specific interactions, such as growth inhibition of competitors (Berry *et al.*, 2008) or enslavement of other organisms (Bar-Yosef *et al.*, 2010). However this hypothesis is an ongoing matter of debate.

Until now, most commercial bioactive compounds have been isolated from freshwater cyanobacteria, the abundance and diversity of which is assumed to be greater than that of marine cyanobacteria. However, marine environments with variable environmental conditions are also potentially good sources for secondary metabolites of cyanobacteria with commercial applications (Burja *et al.*, 2001; Bhadury and Wright, 2004). In particular, some marine cyanobacteria offer the possibility of mass production of vitamins, such as vitamins B and E (Plavsic *et al.*, 2004); other compounds may act as therapeutics, insecticides, and antifouling agents (Burja *et al.*, 2001; Shimizu, 2003; Berry *et al.*, 2008; Abed, Dobretsov, and Sudesh, 2009; Boopathy and Kathiresan, 2010 and references therein).

3.5 Ecophysiology of nitrogen-fixing cyanobacteria

In nitrogen-limited photic environments (especially those lacking nitrate and ammonium), cyanobacteria are the main diazotrophic (nitrogen-fixing) organisms (Zehr, 2011). The process of nitrogen fixation is catalyzed by the enzyme nitrogenase. This consists of two proteins: dinitrogenase reductase encoded by the gene *nifH*, and dinitrogenase, encoded by genes *nifD* and *nifK*. Nitrogen fixation is energetically costly, and the activity of nitrogenase is tightly regulated at both transcriptional and posttranscriptional levels. Since the nitrogenase enzyme is instantaneously and irreversibly inactivated by oxygen, nitrogen fixation occurs in anoxic or minimal oxygen conditions. Diazotrophic cyanobacteria have evolved several strategies with different degrees of complexity to protect their nitrogenase from oxygen, which is present in the surrounding medium and also produced intracellularly (Gallon, 1992). Most cyanobacteria cope with this incompatibility mainly by separating the two processes spatially or temporally, or through a combination of the two. Another strategy, observed in *Plectonema boryanum*, *Oscillatoria limnetica*, and *Synechococcus*

sp., is "avoidance" (of oxygen), expressing nitrogenase only under anaerobic conditions. This strategy works through photosystem II inhibition – by the presence of sulfide or by migrating from the exposed surface layer of a microbial mat towards the lower and anoxic parts, where the far-red light still maintains the function of photosystem I (Villbrandt and Stal, 1996; Misra, 1999).

The most complex and efficient nitrogenase protection strategy is that of spatial separation by differentiation and development of specialized cells for nitrogen fixation, known as "heterocysts." Heterocysts have thick cell walls to reduce oxygen diffusion, and have no photosystem II oxygen production (Adams and Duggan, 1999). Heterocystous cyanobacteria are mainly free-living in fresh and brackish waters, in soils, and also in the marine environment, they mainly occur in symbioses with other organisms. However, why free-living heterocystous cyanobacteria are either absent or rare in marine planktonic and benthic enviroments is still not well understood (see Stal, 2009 for discusion).

For a long time, heterocysts remained the only differentiated cell type for daytime diazotrophy. Diazocytes are semi-differentiated cells performing nitrogen fixation and, so far, have only been encountered in the marine planktonic free-living filamentous *Trichodesmium* (Bergman and Carpenter, 1991; Fredriksson and Bergman, 1997; El-Shehawy *et al.*, 2003). A combination of spatial (diazocytes) and temporal separation strategies (during light periods) has also been reported in *Trichodesmium* (Bergman and Carpenter, 1991; Berman-Frank *et al.*, 2001). Nitrogen fixation peaks at midday, with a concomitant dip in photosynthetic activity (Berman-Frank *et al.*, 2001). Other non-heterocystous diazotrophic cyanobacteria employ a temporal separation strategy only, for example *Lyngbya*, *Cyanothece*, and *Crocosphaera* (Gallon *et al.*, 1988; Bergman *et al.*, 1997). In unicellular *Cyanothece* and *Crocosphaera*, genes that could enable nitrogen fixation during daylight under very low oxygen conditions have been identified (Compaoré and Stal, 2010). It has been suggested that uncultured marine unicellular UCYN-A lack PSII and RuBisCO, allowing the cyanobacterium to fix nitrogen during the daytime without interference from oxygen-generating photosynthesis (Zehr *et al.*, 2008; Tripp *et al.*, 2010).

During the process of nitrogen fixation, hydrogen is produced as a by-product, which is a potential future energy source. Heterocystous cyanobacteria have been reported as the most efficient producers of hydrogen among cyanobacteria (Pinzon-Gamez, Sundaram, and Ju, 2005). Under specific conditions, such as sulfur starvation, complete darkness, and anoxia, some non-heterocystous cyanobacteria, for example unicellular *Gloeocapsa alpicola* (Antal and Lindblad, 2005), *Synechocystis* PCC 6803 (Baebprasert, Lindblad, and Incharoensakdi, 2010), and filamentous *Spirulina platensis* (Aoyama *et al.*, 1997) showed increased hydrogen production.

3.5.1 Marine free-living and symbiotic cyanobacteria

3.5.1.1 Planktonic environments

The importance of marine cyanobacteria in the oceanic nutrient cycles has only been recognized in recent decades (Karl *et al.*, 2002). The planktonic unicellular cyanobacteria *Synechococcus* and *Prochlorococcus* are the quantitatively dominant biomass producers and carbon fixers in the global ocean ecosystem (Liu, Nolla, and Campbell, 1997; Partensky, Blanchot, and Vaulot, 1999a). They account for about half of all marine photosynthesis in the photic zone of the oceans (Chisholm *et al.*, 1988). In particular, *Prochlorococcus marinus* is considered the most abundant photosynthetic cell on earth, mainly occurring in the oligotrophic oceans. It was one of the first marine bacteria to have its genome sequenced (Dufresne, *et al.*, 2003; Rocap *et al.*, 2003). On the other hand, the nutrient-rich upwelling coastal zones are dominated by *Synechococcus* (Partensky *et al.*, 1996; Ahlgren and Rocap, 2006). Both these genera have diverged into various ecotypes down the water column, primarily due to the variation in light and nutrient conditions (Moore, Rocap, and Chisholm, 1998; Rocap *et al.*, 2003). The earlier assumption was that *Prochlorococcus* was unable to assimilate nitrate and only low-light ecotypes could assimilate nitrite. However recent metagenomic analyses, which were subsequently verified with laboratory experiments, showed that *Prochlorococcus* contains genes for both nitrate and nitrate reductases (Martiny, Kathuria, and Berube, 2009). Furthermore, *Prochlorococcus* has also been found to be the major microbial utilizer of phosphonates (organic C–P phosphorus compounds) in the oceans (Feingersch *et al.*, 2012). New findings suggest that small oceanic picoplankton are widespread

and abundant due to a "simple"' survival strategy of maintaining only essential functions and streamlining of their genomes (Yooseph, *et al.*, 2010).

Since open oceans are typically nitrogen limited, nitrogen-fixing cyanobacteria play an important role in these habitats. *Trichodesmium* is a principal nitrogen fixer in open tropical oceans (Capone *et al.*, 1997), where it normally forms "bloom". Amongst the species of *Trichodesmium*, *T. erythraenum* is more frequently encountered than species like *T. theibautii*, *T. hilderbrandi*, and *T. rubescens*. Moreover, nitrogen-fixing unicellular cyanobacteria have also been reported in varied marine habitats, and have been recognized as ecologically important (Zehr *et al.*, 2001; Montoya *et al.*, 2004; Church *et al.*, 2005b; Langlois, LaRoche, and Raab, 2005; Foster, Carpenter, and Bergman, 2006; Moisander *et al.*, 2010). A study of sea water and sea ice in the Arctic Ocean suggests a wider distribution of these marine nitrogen-fixing cyanobacteria than previously reported, further underlining their potential importance in the global nitrogen cycle (Díez *et al.*, 2012).

Heterocystous cyanobacteria are conspicuously absent in the tropical oceans. Their distribution is suggested to be restricted by temperature (Staal, Meysman, and Stal, 2003). However, there are examples of brackish ecosystems where heterocystous cyanobacteria are of great ecological importance, for example the Peel-Harvey Estuary in Western Australia and the Baltic Sea. Large summer blooms of toxic *Nodularia spumigena* characterize these water bodies. In the Baltic Sea the cyanobacterium co-occur with *Aphanizomenon* sp. and *Anabaena flos-aquae* (Laamanen *et al.*, 2001; Laamanen, Forsström, and Sivonen, 2002).

Marine cyanobacteria are important partners (in symbiosis) for many eukaryotes, ranging from microscopic diatoms to marine angiosperms. For example, the filamentous heterocystous species *Richelia intracellularis* and *Calothrix rhizosolenia* live intracellularly in diatoms, typically in genera *Climacodium*, *Hemiaulus*, *Chaetoceros*, and *Rhizosolenia* (Janson *et al.*, 1999; Carpenter and Janson, 2000; Foster and Zehr, 2006). Transfer of nutrients between symbionts has been quantified and visualized using nanometer-scale secondary ion mass spectrometry, indicating that cyanobionts provide fixed nitrogen to their diatom host (Foster *et al.*, 2011). This suggests that cyanobacteria–diatom symbioses could be quantitatively as important as contributors of nitrogen in the open ocean as free-living *Trichodesmium* (Villareal, 1991; Carpenter *et al.*, 1999; Foster, Subramaniam, and Zehr, 2009; Goebel *et al.*, 2010).

3.5.1.2 Benthic environments

Cyanobacteria found in marine intertidal and littoral ecosystems play a major role in stabilizing near-shore sediments (Noffke *et al.*, 2003). Interestingly, and for unknown reasons, marine benthic cyanobacteria have wider morphological and genetic diversity than their planktonic counterparts. Some marine fossilized and ancient living stromatolites dated at around 3.4 billion years old are believed to have been generated by cyanobacteria (Stal, 2000; Steppe *et al.*, 2001). Phylotypes related to varied unicellular (e.g., *Euhalothece*, *Gloeocapsa*, *Gloeothece*, *Chroococcidiopsis*, *Dermocarpella*, *Acaryochloris*, *Synechococcus*, and *Gloeobacter)* as well as filamentous, non-heterocystous (e.g., *Arthospira*, *Geitlerinema*, *Plectonema*, *Schizothrix*), and heterocystous (*Nostoc* and *Fischerella* spp.) genera have been described as the main cyanobacteria present in marine and hypersaline stromatolites (Golubic, 1991; Neilan *et al.*, 2002; Foster and Green, 2011). However, studies of stromatolites at Shark Bay, Western Australia suggest that other bacteria, such as alphaproteobacterial anoxygenic phototrophs, can also be important populations in these ancient habitats (Papineau *et al.*, 2005; Goh *et al.*, 2009).

Free-living benthic cyanobacteria can occur in a wide variety of coastal habitats, including tropical and temperate sand flats, mangrove forests, seagrass meadows, splash zones of rocky shores, as well as on other biological or artificial benthic coastal substrates. These benthic cyanobacteria have been less studied than planktonic species. However, studies suggest that these habitats have diverse cyanobacterial communities, including nitrogen-fixing genera (Stal, 2000; Charpy-Roubaud, Charpy, and Larkum, 2001; Díez, Bauer, and Bergman, 2007; Charpy *et al.*, 2007; Bauer *et al.*, 2008; Severin and Stal, 2010; Severin, Acinas, and

Stal, 2010). These communities are typically a mix of species from all major cyanobacterial clades, and morphotypes range from unicellular to filamentous and heterocystous species. Different species typically coexist in microbial mats of varying thickness. The most common species are *Lyngbya majuscula*, *Microcoleus chthonoplastes*, and different species of *Oscillatoria* (Paerl, Fitzpatrick, and Bebout, 1996; Bauer *et al.*, 2008; Severin and Stal, 2008). These mats can be stratified and exhibit steep gradients in physicochemical parameters such as pH and oxygen. Many studies of microbial mats describe nitrogen-fixation activity but this is not always due to cyanobacteria since these mats also contain a wide range of heterotrophic bacteria (Zehr *et al.*, 1995). Recently, evidence of horizontal gene transfer of *nifH* genes from bacteria to the very common mat-forming non-heterocystous *Microcoleus chthonoplastes* suggests that attributing a function to a taxon based on analyses of single genes in a complex community can be ambiguous (Bolhuis *et al.*, 2010).

The marine unicellular cyanobacteria *Prochloron* spp. and *Acaryochloris marina* both live in symbiosis with benthic eukaryotic organisms such as ascidian (tunicate) species (Lewin and Cheng, 1989; Miyashita *et al.*, 1996). There are also examples of cyanobacteria living in close association with seagrasses (Uku *et al.*, 2007; Hamisi *et al.*, 2009). Both filamentous (non-heterocystous and heterocystous) and unicellular species have been reported living epiphytically on seagrass phyllosphere. It is still unclear if there is any nutrient transfer in this association but nitrogen-fixation rates and expression of cyanobacterial *nifH* genes have been reported from seagrass beds in the western Indian Ocean (Hamisi *et al.*, 2013, manuscript in review). Cyanobacteria have also been reported associated with corals (Charpy *et al.*, 2012 and references therein). In particular, cyanobacteria with diazotrophic potential have been reported as epyphites (Hewson *et al.*, 2007) and as symbionts in the coral *Montastraea cavernosa* (Lesser *et al.*, 2004, 2007). Marine sponges can also host a variety of cyanobacteria (Tang *et al.*, 2011). Finally, nitrogen-fixing activity of cyanobacteria has also been documented on mangrove roots (pneumatophores) (Lugomela and Bergman 2002b). However, the nature of the relationship (clearly non-pathogenic) has still to be further explored.

3.5.2 Terrestrial and freshwater cyanobacteria

3.5.2.1 Free-living organisms

Free-living cyanobacteria are common inhabitants of freshwater systems worldwide. In freshwater ecosystems, the occurrence of blooms of cyanobacteria is usually a sign of eutrophication. Their rapid response and sensitivity to changes in environmental conditions make cyanobacteria potentially good biological indicators of pollution or other environmental changes. In particular, nitrogen-fixing cyanobacteria have often been used as biosensors to estimate water quality (especially high turbidity), low N:P ratio, metal toxicity, and nitrogen limitation in the environment. The unicellular *Microcystis* spp., *Synechocystis* spp., and *Synechococcus* spp., the filamentous heterocystous *Aphanizomenon* spp., *Anabaena* spp., and *Cylindrospermopsis raciborskii*, as well as the non-heterocystous *Planktothrix* spp., *Lyngbya* spp., and some *Oscillatoria* spp. are among the most common species that can be used as biological indicators. Nutrient-enriched wastewater and industrial effluents can provide an ideal environment for cyanobacteria (Vasconcelos and Pereira, 2001), and they have been successfully used for bioremediation of polluted water (Lincoln, Wilkie, and French, 1996; Dubey *et al.*, 2011). Some cyanobacteria isolated from pharmaceutical and textile industry effluents are also capable of biodegradation and biosorption of heavy metals (Dubey *et al.*, 2011) and melanoidin pigment (Agarwal *et al.*, 2010). Finally, many of these freshwaster cyanobacteria can also produce potent toxins, causing health problems and economic losses.

3.5.2.2 Symbioses

The majority of cyanobacterial symbioses and associations have been found in terrestrial environments. Most symbioses and associations involve nitrogen fixation, and, depending on the host, cyanobacterial photosynthesis may be reduced or redundant in the partnership. Cyanobacterial diazotrophs provide nitrogen to the ecosystem in which they live, and often the rates of fixation by these systems are higher than those of free-living cyanobacteria, perhaps due to

the additional nutrition and micro-aerobic conditions provided by the host. The ability of cyanobacteria to fix nitrogen either independently or in symbiosis with other organisms not only contributes to natural ecosystems but also has economic applications (e.g., in rice and cereal fields and in general as fertilizers of many other soils for agriculture).

Some of the best-studied terrestrial symbioses include cyanolichens, often pioneering colonizers of bare soils, rocks, and other exposed surfaces with few nutrients. These can produce abundant growth in wetlands, marshy lands, and rice fields, where the *Nostoc–Gunnera (*angiosperms) and cyanobacteria–Cycad (in their coralloid roots) symbioses can also be abundant (Rai, Bergman, and Rasmussen, 2002). In recent years, cyanobacteria living in association with feather mosses in boreal forests, the world's largest terrestrial ecosystem, have been recognized as important nitrogen fixers (DeLuca *et al*., 2002; 2008). These forests harbor copious amounts of biomass in the form of coniferous trees, yet soils are typically poor in nutrients. In areas with a substantial forest industry these ecosystems are of great financial as well as ecological importance. Feather mosses, which cover approximately 80% of the boreal forest floor, have associated heterocystous cyanobacteria growing on stems and leaves. The cyanobacteria are typically different types of *Nostoc*, but heterocystous *Stigonema* and *Calothrix* (Gentili *et al*., 2005) have also been reported, and both *Nostoc* and *Cylindorspermum* have been isolated in cultures from moss samples collected in northern Sweden (Ininbergs *et al*., 2011).

As nitrogen is one of the most important macronutrients and is widely used in fertilizers, the use of biological nitrogen fixation (diazotrophy) in agriculture represents a potential area for further exploitation, by traditional means but also by use of synthetic biology approaches. There have indeed been some successful attempts to establish artificial associations between nitrogen-fixing cyanobacteria and plant hosts of economic importance, such as cereals (Gusev *et al*., 2002).

Cyanobacterial symbioses also occur in freshwater environments. The waterfern *Azolla*, with its obligate symbiotic nitrogen-fixing heterocystous filamentous cyanobacterial partner (*Anabaena azollae)* has historically been used as a biofertilizer in ricefields. The taxonomy of the cyanobiont has been under debate and it has been shown that different strains inhabit different host partners (Papaefthimiou *et al*., 2008). This symbiosis has been particularly challenging to study since the cyanobiont cannot be grown outside of its host and is vertically transmitted through the *Azolla* sporocarp, although it resides extracellularly in the leaf cavity of the fern. The genome of the cyanobiont was recently sequenced, and revealed an unusually large number of tranposases and a reduction in genome size compared to other filamentous cyanobacteria. Based on this observation, it was suggested that this symbiosis represents the initial phase of an endosymbiotic event that possibly could result in a nitrogen-fixing plastid (Ran *et al*., 2010).

3.5.3 Free-living cyanobacteria in extreme habitats

Cyanobacteria have high adaptability and can inhabit extreme habitats, including those with high (thermophiles) and low (psychrophiles) temperatures, high salt concentration (halophiles), and high radiation. Cyanobacteria endure these extreme conditions through a number of protective strategies that make them highly competitive. They have storage and other mechanisms (e.g., nitrogen fixation) to deal with nutrient deficiency, light-capturing systems and photoprotective quenching pigments, as well as temperature-tolerance mechanisms (Tang, Tremblay, and Vincent, 1997; Hawes and Schwarz, 2001; Vincent, 2007; Zakhia *et al*., 2008). This flexibility and capacity of adaptation to extreme conditions make them good candidates to produce materials of interest for biotechnology and industrial processes.

3.5.3.1 Cyanobacteria living at high temperature in hot springs

Temperature is one of the main factors determining the distribution and abundance of species due to its effect on enzymatic activities (Alexandrof, 1977). High-temperature environments are considered as hot-spots in which to search for new organisms with biotechnological applications. In particular, hot springs are unique ecosystems where most eukaryotes cannot survive, making bacteria and archaea the dominant organisms, and cyanobacteria are particularly abundant. Cyanobacteria occur mostly in alkaline hot

springs (Miller, Wingard, and Castenholz, 1998). They were first described in Yellowstone National Park, Wyoming, USA (Brock, 1978; Castenholz, 1969) and were later found in geothermal areas all over the world (Ward and Castenholz, 2000; Papke *et al.*, 2003; Berrini *et al.*, 2004; Sompong *et al.*, 2005; Phoenix *et al.*, 2006; Miller, Castenholz, and Pedersen, 2007).

In hot springs cyanobacterial diversity varies with temperature, as the effluent water cools down with increasing distance from the source. At lower temperatures (40–55°C), filamentous cyanobacteria dominate the microbial mats (Ward and Castenholz, 2000; Lau *et al.*, 2005). Filamentous cyanobacteria like *Mastigocladus laminosus*, *Phormidium* spp. and different *Oscillatoria* species have their upper temperature limit for growth between 55 and 62°C. At the highest temperatures (> 65°C and up to 74°C), unicellular cyanobacteria of the genus *Synechococcus* (*Thermosynechococcus*) appear to be better adapted in both planktonic and benthic habitats (Ward *et al.*, 1998; Steunou *et al.*, 2006, 2008).

Nitrogen fixation in hot springs has been mainly attributed to filamentous heterocystous cyanobacteria or heterotrophic bacteria (Stewart, 1970; Wickstrom, 1980; Thomsen and Cox, 1993). The fact that microbial mats in these systems can become highly saturated with oxygen during daytime probably only allows diazotrophy by heterocystous cyanobacteria, since non-heterocystous cyanobacteria usually require a dark period for this anaerobic process (Stal, 2000). The true branching heterocystous cyanobacteria Stigonematales have been described as the largest and most frequent constituent of microbial mats in hot springs (Castenholz, 1969; Miller, Purugganan, and Curtis, 2006; Miller, Castenholz, and Pedersen, 2007). Active nitrogen fixation has been observed in the termophilic genus *Mastigocladus* above 55°C, with an optimum temperature close to 43°C (Castenholz, 1969; Miyamoto, Hallenbeck, and Benemann, 1979). The *nif* genes have also been identified in two thermophilic *Synechococcus* ecotypes (*Synechococcus* OS-A and OS-B), abundant in microbial mats in the Yellowstone hot springs at high temperatures (>55°C) (Steunou *et al.*, 2006). Nitrogen fixation by *Synechococcus* OS-A and OS-B in anoxic mats during the night has been suggested (Steunou *et al.*, 2006, 2008). Steunou *et al.*, (2006, 2008) suggest that at night, fermentation pathways in *Synechococcus* generate enough energy to support fixation.

Many industrial processes involve high temperatures (above 45°C), which cause problems for most enzymes. Thermostable enzymes recovered from bacteria inhabiting hot springs are of wide use in industrial and medical applications. Cyanobacterial production of thermostable enzymes (polymerases, proteinases, amylases, xylanases) has been reported (Piechula *et al.*, 2001). In addition, Sato *et al.*, (2007) described the successful production of D-amino acid dipeptides in an ATP regeneration system at high temperature by using a thermostable polyphosphate kinase from the thermophilic cyanobacterium *Thermosynechococcus* elongatus BP-1.

3.5.3.2 Cyanobacteria inhabiting low-temperature ecosystems

Low-temperature ecosystems also generate stress, forcing organisms to develop adaptations. The polar regions and other cold habitats are extreme environments from which important contributions have been made to biotechnology through exploitation of their cyanobacteria. Like other organisms, cyanobacteria can adjust their membrane fluidity to function at low temperatures by regulation of their lipid composition. However, until now, just a few cyanobacteria have been considered true psychrophiles (Tang, Tremblay, and Vincent, 1997; Fritsen and Priscu, 1998), and most are classified as psychrotolerant, capable of metabolizing near 0°C but typically having their growth temperature optimum above 15°C. The latter are adapted to freeze/thaw survival, photoprotection, and acclimation of their light procurement mechanisms for photosynthesis (Nadeau, Howard-Williams, and Castenholz, 1999; Vincent, 2007).

Cyanobacteria often produce conspicuous crusts, mats, and biofilms on substrates such as snow, ice, soil, and rocks where they represent the main primary producers (Moorhead and Priscu, 1998; Elster and Komárek, 2003). Viable cyanobacteria are also found as a common constituent of permafrost (Vishnivetskaya *et al.*, 2005), deglaciated soils (Kastovska *et al.*, 2005), and on the inside and outside of rocks (Friedmann, Hua, and Ocampo-Friedmann, 1988; Cockell and Stokes, 2004) in cold regions. One of the most common cyanobacterial genera observed in these cold habitats is the unicellular cyanobacterium *Chroococcidiopsis* (Vincent, 1988, 2000; Grilli-Cailola and Billi,

2007; Bahl *et al.*, 2011). However, a wide range of species, mainly free-living filamentous species such as *Nostoc* and Oscillatoriales have also been found growing in the water column, mats, and ice in Antarctic lakes (Wharton, Parker, and Simmons, 1983; Olson *et al.*, 1998; Taton *et al.*, 2006; Singh and Elster, 2007), and in the Arctic (Vézina and Vincent, 1997; Vincent and Hobbie, 2000; Jungblut, Lovejoy, and Vincent, 2010). Moreover, planktonic picocyanobacteria may constitute about 80% of the photosynthetic organisms in some lakes in Antarctica (Vézina and Vincent, 1997; Powell *et al.*, 2005) and in the coastline of high Arctic Canada (Van Hove *et al.*, 2006). Cyanobacteria can also be very abundant in shelf ice ponds in the Arctic (Vincent, Mueller, and Bonilla. 2004; Mueller *et al.*, 2005, Jungblut, Lovejoy, and Vincent, 2010) and in Antarctica (Jungblut *et al.*, 2005, Jungblut and Neilan, 2010), as well as in many rivers and streams (Vincent *et al.*, 1993; Elster *et al.*, 1997; McKnight *et al.*, 1999; Fernández-Valiente *et al.*, 2001, 2007).

Furthermore, cyanobacteria from the order Chroococcales, Oscillatoriales and Nostocales have been described as an important source of nitrogen input in terrestrial (mostly in symbiosis with mosses and lichens) and in polar freshwater environments (Chapin and Bledsoe, 1992; Liengen, 1999; Fernández-Valiente *et al.*, 2001) as well as in alpine glaciers (Duc *et al.*, 2009). Heterocystous species of the genus *Nostoc* have been reported as a potentially important nitrogen fixer in the McMurdo ice shelf (ponds) in Antarctica (Jungblut and Neilan, 2010).

On the other hand, cyanobacteria are believed to be relatively rare in polar marine water (Waleron *et al.*, 2007; Koh *et al.*, 2012). However, recently Díez *et al.*, (2012) demonstrated the presence of *nifH* genes, which are found in diverse groups of cyanobacteria with the previously demonstrated ability to fix nitrogen, in samples from Arctic seawater and hypersaline localities in the ice (brines). Some of these might be related to cyanobacteria found in tropical and temperate regions. Particularly in the ice brine, unicellular cyanobacteria are well represented and could be the main diazotrophs in these systems (Díez *et al.*, 2012).

Cyanobacteria recovered from these frozen environments have many different industrial applications. Their cold-adapted enzymes can be used for perfumes and other cosmetics (most do not tolerate low temperatures), as well as detergents to wash in cold water, antifreezes, and so on.

3.5.3.3 Cyanobacteria inhabiting highly acidic or alkaline habitats

Filamentous cyanobacteria such as *Spirulina* sp. have been found in acidic Bavarian lakes, even at pH of around 2.9. The unicellular genera *Aphanocapsa* and *Chroococcus* have been found to dominate in acidified Canadian lakes with pH below 3.7 (Steinberg, Schafer, and Beisker, 1998). Acid-stress-tolerant proteins have been reported for the nitrogen fixer *Nostoc*, which makes them good candidates for increasing productivity in diverse pH rice-field soils (Karthikeyan and Gopalaswamy, 2009).

In contrast, because carbon dioxide uptake during photosynthesis leads to an increase in pH, cyanobacteria can also be exposed to high pH during the daytime. Cyanobacteria (e.g., *Spirulina platensis*, *Anabaenopsis*, *Synechococcus*, *Gloeocapsa*) have been shown to be adapted to high alkaline environments in the soda lakes of East Africa (Grant and Tindall, 1986; Boussiba and Zarka, 2000; Oren and Seckbach, 2001). In particular, *Plectonema nostocorum* has been reported to occur up to pH 13, the highest pH value reported to sustain living organisms.

3.5.3.4 Cyanobacteria living under high radiation

As exposure to high solar radiation in extreme habitats constitutes a stress for cyanobacteria (as for any other organism), they have developed extremely thick external sheath layers (EPS) to better withstand desiccation and ultraviolet light (Helm *et al.*, 2000). In *Nostoc commune*, the EPS is described as a crucial protectant against UVB, desiccation, and freeze and thaw to maintain the integrity of its membrane (Hill *et al.*, 1997; Tamaru *et al.*, 2005).

In cyanobacteria another adaptation to UVB exposure is *de novo* synthesis of less-sensitive isoforms of the D1 and D2 polypeptides at the heart of the photosystem II (PSII) reaction center (Gao and Garcia-Pichel, 2011). In planktonic and benthic environments, many cyanobacteria migrate in response to UV radiation (Quesada and Vincent, 1997; Nadeau, Howard-Williams, and Castenholz, 1999). *Microcoleus*

chthonoplastes have been shown to exhibit a greater incidence of migration in response to UVB radiation than UVA and PAR (Bebout and Garcia-Pichel, 1995). In hot and cold deserts, cyanobacteria are common in cracks and the underside of translucent rocks, where radiation exposure is highly attenuated (Wynn-Williams, 2000; Cockell and Stokes, 2004). In particular, hypolithic communities containing unicellular *Chroococcidiopsis* morphospecies have been reported from all over the world (Bahl *et al.*, 2011). For example, they colonize translucent stones in the Atacama desert in Chile (Wierzchos, Ascaso and McKay, 2006). Moreover, most soil crusts are dominated by cyanobacteria, which are of great importance to trace water in desert and semi-desert regions (Garcia-Pichel and Pringault, 2001; Maestre *et al.*, 2006; Viles, 2008). Deep ice-covered lakes and thick biofilms also provide refugia from the damaging effects of radiation. In some alkaline hot springs, filamentous cyanobacteria have been found to be encased in an amorphous silica matrix (sinter surface) that attenuates UVB while permitting minimal PAR levels to penetrate (Phoenix *et al.*, 2006). All these studies demonstrate that cyanobacteria in their natural environment and as part of the microbial consortia develop strategies against desiccation and high radiation levels.

To scavenge UVB-induced ROS formation, cyanobacteria synthesize and accumulate non-enzymatic antioxidants such as carotenoids, tocopherols, ascorbic acid, and reduced glutathione. However, the most famous "sunscreen" compound found in more than 300 cyanobacterial species inhabiting intertidal mats, epilithic biofilms, and biological soil crusts is scytonemin (Garcia-Pichel and Castenholz, 1991; Gao and Garcia-Pichel, 2011). As examples, the filamentous genera *Scytonema*, *Nostoc*, *Lyngbya*, and *Calothrix* (Dillon, Miller, and Castenholz, 2003; Balskus, Case, and Walsh, 2011) and the unicellular *Chlorogloeopsis* sp. (Garcia-Pichel, Sherry, and Castenholz, 1992) and *Chroococcidiopsis* sp. (Dillon *et al.*, 2002) accumulate scytonemin in the sheath surrounding the cells (Castenholz and Garcia-Pichel, 2000), preventing 90% of incident UVA from entering the cell (Garcia-Pichel, Sherry, and Castenholz, 1992).

Many cyanobacteria also synthetize and accumulate mycosporine-like amino acids (MAAs) after UVB induction (Balskus and Walsh, 2010, and references therein). In most cyanobacteria, MAAs are located in the cytoplasm, and cells that accumulate higher concentrations are estimated to be approximately 25% more resistant to UVB than those with lower levels (Garcia-Pichel and Castenholz, 1993). More than 20 different MAAs from cyanobacteria have been described. The most common MAAs are shinorine, asterina-330, porphyra-334, and mycosporine-glycine.

3.5.3.5 Cyanobacteria inhabiting high saline ecosystems

Many cyanobacteria isolated from coastal environments have been found to tolerate saline environments (i.e., they are halotolerant) rather than requiring salinity (i.e., halophilic). However, cyanobacteria also frequently colonize euryhaline (very saline) environments, and are capable of growth at combined salt concentrations as high as 3–4 molar mass (Oren, 2010). Halophilic cyanobacteria contribute to the primary production in salt lakes, hypersaline lagoons, and solar salterns (Golubic, 1980; Oren, 2002). The most widely distributed species are the filamentous *Microcoleus chthonoplastes* (Whitton and Potts, 2000; Oren, 2002) and *Halospirulina tapeticola* (Nübel, Garcia-Pichel, and Muyzer, 2000), and the unicellular *Aphanothece halophytica* (Garcia-Pichel, Nübel, and Muyzer, 1998). Halophilic and halotolerant cyanobacteria manage to maintain an osmotic equilibrium with the environment by lowering their intracellular ionic concentration levels or accumulating compatible low-molecular-weight solutes. In cyanobacteria the types of the osmotic solutes they use can vary depending on the organism. Accumulation of disaccharides such as sucrose and trehalose is used to modulate osmotic equilibrium in the case of less tolerant strains such as *Chroococcidiopsis* (Hershkovitz, Oren, and Cohen, 1991), glucosylglycerol in *Synechocystis* sp. PCC 6803 (Hagemann and Erdmann, 1994), and glycine betaine and L-glutamate betaine in strains adapted to the highest salt concentrations, such as *A. halophytica*, *Halospirulina* (Mackay, Norton, and Borowitzka, 1984; Incharoensakdi and Wutipraditkul, 1999). The latter halotolerant cyanobacteria also remove the excess of Na^+ from their cells so as to maintain the cytoplasmic Na^+ concentration by employing an Na^+-ATPase (Wiangnon, Raksajit, and Incharoensakdi, 2007). The same mechanism is also found in *Anabaena* sp. PCC 7120 (Neisser *et al.*, 1994). In addition, some halophilic

cyanobacteria accumulate unusually large concentrations of MAA, compounds that have been suggested to also have a role as compatible solutes (Oren, 1997).

3.6 Concluding remarks

As discussed above, cyanobacteria inhabit all possible habitats, performing crucial ecological services. However, to date, no quantitative assessment is available on the monetary value of the services rendered by cyanobacteria in their respective habitats. However, genomic and metagenomic analyses of environmental cyanobacterial communities will further advance functional interpretations and elucidate the biotechnological potential of these resilient and adaptable organisms. Up to today, comparative analyses of cyanobacteria have revealed novel metabolic pathways, exclusive protein families, and frequent lateral-gene transfer events (Mulkidjanian *et al.*, 2006; Zhaxybayeva *et al.*, 2006). These approaches will continue to shed light on little-studied habitats and species, and further deepen our knowledge of the diversity and ecology of cyanobacteria, and their potential for commercial expoitation.

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Chapter 4
Cyanobacteria and carbon sequestration

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4.1 Introduction

Carbon dioxide is the main gas causing the greenhouse effect because of its large emissions and its climate-altering potential. There are many technologies available for carbon capture and storage, the aim of which is to stabilize carbon dioxide emissions and to earn carbon credits. However, most of these technologies are not technically and economically feasible for large-scale application. According to the Intergovernmental Panel on Climate Change (IPCC, 2007), the choice of mitigation technologies is dependent on factors such as cost, capture capacity, environmental impact, and speed of introduction of the new technology as well as social factors, for example public acceptance. In this sense, the use of biochemical engineering processes for carbon dioxide sequestration is a potential technology to decrease emissions of this gas. These carbon dioxide sequestration processes are based on photosynthetic reactions in bioreactors, where microalgae act as biocatalysts in a series of biochemical reactions responsible for the bioconversion of carbon dioxide into photosynthetic metabolic products.

4.2 Carbon dioxide emission sources

On a global basis, it is estimated that approximately 30,000,000 thousand metric tons are emitted annually as a result of man's activities. These emissions are the result of stationary, mobile, or natural sources of carbon dioxide (Dhillon and Wuehlisch, 2013).

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Stationary sources are mainly industrial processes. Electrical energy generating plants fueled by fossil fuels, hydrogen and ammonia production plants, cement factories, and fermentative and chemical oxidation processes are the largest contributors to carbon dioxide emissions worldwide. In addition to the carbon dioxide emitted industrially, the carbon dioxide generated in residences, buildings, and commercial complexes also contributes to stationary emissions, as do forest and agricultural fires. The sources of mobile emissions are mainly passenger and cargo transport, including cars, trucks, buses, planes, trains, and ships. Human and animal metabolism, plant and animal degradation, and volcanic and oceanic activity are the main natural sources of carbon dioxide. Sources of anthropogenic emissions include stationary and mobile sources but exclude natural sources (Song, 2006).

Stationary industrial emissions are the most suitable for application of biotechnology sequestration processes because of the transport characteristics of the gaseous streams required in the injection systems of bioreactors. Another limiting factor when choosing the source of carbon dioxide to mitigate is the temperature and composition of the emission sources. In most cases, the temperature of gaseous streams is several times the limit tolerated by most microorganisms. Biologically mediated processes require cold gases, which are rarely observed in conventional industrial flue gases, which can reach thousands of degrees Celsius, limiting the use of biotechnology on most sources of industrial gases. Additionally, as well as being rich in carbon dioxide, industrial gas streams are rich in other constituents, for example particulate matter, hydrogen, methane, carbon monoxide, nitrous oxide, sulfur dioxide, nitrogen, oxygen, and others. Isolated or combined, these constituents have a toxic and/or inhibitor effect on cyanobacteria growth and directly impact bioreactor performance. Although it is thought that there is high availability of industrial carbon dioxide, in practical terms this is not true when use in cyanobacteria-based systems is considered (Jacob-Lopes and Franco, 2009). According to these authors, the cooling of gas streams associated with stream dilution or pre-adaptation of microorganisms producing tolerant mutants, are the main strategies adopted to break through these initial barriers.

4.3 Importance of cyanobacteria in carbon sequestration

Cyanobacteria include approximately 150 genera and 2000 species, distributed in various natural environments. Many of these environments are extreme, allowing the selection, adaptation, and growth of organisms that are highly tolerant to adverse conditions. Biotechnological exploration of the metabolic routes of these species is therefore more likely to meet with success (Subramanian and Thajuddin, 2005).

Cyanobacteria are the only prokaryotes that perform a plant-like oxygenic photosynthesis. They show great versatility in maintaining their structures, using different energy-generating metabolisms such as respiration and nitrogen fixation, in addition to photosynthesis (Queiroz *et al.*, 2007). Cyanobacteria have high content of pigments – mainly chlorophyll *a*, and phycobiliproteins (phycocyanin, phycoerythrin, and allophycocyanin) – that are involved in light energy harvesting. The spectral light-absorption characteristics of cyanobacteria are different from those of other photosynthetic organisms. Photosynthetic activity is supported by light in the spectral region from 665–680 nm, where chlorophyll *a* absorbs light, and also between 620 nm and 560 nm, where light is absorbed by phycocyanin and phycoerythrin, respectively. These features have improved the photosynthetic capacity of cyanobacteria greatly, making them potential biocatalysts for photosynthetic bioprocesses (Grossman *et al.*, 1994).

4.4 Photosynthetic metabolism

The Calvin–Benson–Bassham cycle (reductive pentose phosphate cycle) is the metabolic pathway that governs carbon dioxide fixation in cyanobacteria. It is a two-stage process: a photochemical reaction that uses light to produce biochemical energy and reducing equivalents, and a carbon-fixation reaction that

requires energy and reducing equivalents. Carbon dioxide is incorporated into ribulose-1,5-diphosphate, catalyzed by the enzyme ribolose-1,5-bisphosphate carboxylase oxygenase (RuBisCO). The reaction product is broken into two molecules of phosphoglyceric acid (PGA, a three-carbon molecule), which is reduced in turn by the electron transporter nicotinamide adenine dinucleotide phosphate (NADPH), passing through a series of intermediary phosphorylated sugars, and finally resulting in the production of a glucose (Calvin and Benson, 1948).

The carbon-dioxide fixation (photosynthetic dark reaction) is indirectly a light-dependent process. The demands for energy in the form of ATP and NADPH during various steps of transformations of the Calvin–Benson–Bassham cycle are satisfied in cyanobacteria (as in other oxygenic phototrophs) entirely by photochemical reactions, which occur in the thylakoid, intracytoplasmatic membranes and in one case the cytoplasmic membrane. The electromagnetic sunlight (energy) is absorbed by photosystems I and II, which are highly organized structures of the photosynthetic pigments and electron transporters. The photosynthetically excited electrons flow through an electron-transport chain generating a chemiosmotic potential across the membrane, which is used for the synthesis of ATP, eventually resulting in the formation of electronegative electron transporters such as ferredoxin and NADPH. The source of electrons for oxygenic photosynthesis is water, which, after photolysis (light-driven breaking of water), gives up protons and electrons, with the release of oxygen as a by-product (Campbell *et al.*, 1998; Fromme *et al.*, 2006).

Apart from the Calvin–Benson–Bassham cycle, there is another variant of the carbon dioxide incorporation pathway in cyanobacteria, which is catalyzed by the enzyme phosphoenolpyruvate (PEP) carboxylase. The presence of two carboxylation systems (i.e., RuBisCO and PEP carboxylation) operating in parallel is an important biological feature for the technological application of carbon dioxide sequestration by cyanobacteria. Cyanobacteria use different pathways for inorganic carbon assimilation, aiming to accumulate inorganic carbon actively in order to achieve a satisfactory rate of carbon dioxide fixation. Currently, five different uptake systems operate in cyanobacteria, including three HCO_3^- transporters and two carbon dioxide uptake systems. These complex processes are mediated by an essential biophysical mechanism known as the carbon concentration mechanism, which pumps carbon dioxide to the carboxylation site (the carboxysome), raises intracellular inorganic carbon levels, and compensates for limitations in the carbon dioxide supply (Price *et al.*, 2008). Kaplan *et al.* (2008) have given a detailed account of mechanisms and evolution of carbon acquisition in cyanobacteria.

4.5 Photobioreactors

A photobioreactor is defined as an illuminated culture vessel designed for controlled bioconversion of carbon dioxide into photosynthetic products. A variety of photobioreactors has been developed for cyanobacteria-based processes. Broadly, these can be classified into open and closed photobioreactors (Molina Grima *et al.*, 1999; Merchuk, Gluz, and Mukmenev, 2000). Refer to Chapter 17 for detail on photobioreactors.

Most open systems are natural lakes or open ponds. There are two types of open ponds:

- circular ponds with a rotating arm
- long-channel ponds, either single or connected to each other, and equipped with paddle wheels for mixing; this configuration is commonly known as a raceway pond.

Sunlight falls on the reactor surface and is absorbed by the culture. The photon flux decreases with increasing depth. As a result, bioreactor performance becomes poor because little light energy is available at depth. On the other hand, light intensities are very high close to the reactor surface, causing photoinhibition and/or photosaturation, thereby limiting the performance of the system. Besides these limitations, open photobioreactors have poor mass transfer, no control over water temperature and light conditions, suffer from high evaporative losses, and are vulnerable to contamination. Nevertheless, open systems can be a successful concept because they are inexpensive to build, simple to operate, and easy to scale-up.

The above-mentioned drawbacks can be effectively overcome by choosing adequate locations and species that can grow in selective environments. Raceway ponds are the type frequently used on a commercial basis (Sheehan *et al.*, 1998).

Many species cannot be grown in open ponds because they are easily overgrown by other microorganisms. Therefore, fully controlled closed systems have been devised. Tubular photobioreactors are easy to construct, and have higher volumetric productivities than open ponds. They consist of many tubes, with internal diameter ranging from 3 to 10 cm and lengths between 25 and 100 m. The path that light has to travel is proportional to the diameter of tube. The main drawbacks of the system are high investment and operation costs and poor mass transfer, which can lead to oxygen build-up in the reactor to inhibitory levels, inhibiting the growth of the cyanobacterium (Slegers *et al.*, 2011).

Flat-panel photobioreactors are also too expensive to be economically viable. However, innovative ways to construct lower-cost flat panels are being developed – for example plastic bags in metal racks – and these may be able to make this reactor configuration an industrial reality (Slegers *et al.*, 2013).

Closed photobioreactors are used mainly for biomass production, especially high-value bioproducts. Carbon dioxide sequestration is limited. Losses of 70% of the carbon dioxide injected are typical in conventional photobioreactors receiving high concentrations of carbon dioxide. Carbon dioxide absorption is mainly a function of the volumetric mass transfer coefficient, mass transfer driving force and gas retention time. In this sense, the losses can be limited by means of variations in the operational mode of the photobioreactors. Systems with air recirculation and sequential reactors can improve carbon sequestration in photobioreactors (Jacob-Lopes *et al.*, 2009).

4.6 Biotransformations of carbon dioxide in photobioreactors

In photobioreactors, carbon dioxide is converted by cyanobacteria through chemical and biochemical processes along at least four different paths (Figure 4.1). In the first step, the carbon dioxide dissolved in the aqueous phase of the system can be sequestered by chemical precipitation due to the reaction of the bicarbonate ions and carbonate with elements present in the culture medium, such as calcium and magnesium. Precipitation occurs because an alkaline environment develops during microalgal growth (Lee, Apel, and Walton, 2006).

During photosynthesis carbon atoms coming from the carbon dioxide are incorporated into organic matter, with the help of energy and reducing equivalents provided by photosynthetic electron transport. This conversion of carbon dioxide results in production of cellular biomass, biopolymers dissolved in the culture medium, and volatile organic compounds (VOCs). The conversion of carbon dioxide into biomass is high under a low carbon dioxide mass-loading rate. At high carbon dioxide mass-loading rates, exopolymer

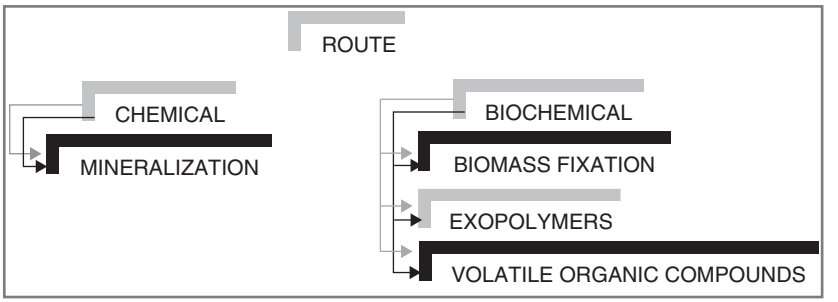

Figure 4.1 Carbon dioxide biotransformation routes in photobioreactors.

and VOC formation are the main carbon dioxide biotransformation routes (Jacob-Lopes *et al.*, 2010).

The secretion of biopolymers into culture media and the release of VOCs are two important routes of carbon dioxide bioconversion in photobioreactors. Cells of cyanobacteria can be single or aggregated into irregular colonies held together by mucilage. The cells, either forming a capsule or sheath in which the cells are embedded, or released into the environment as colloidal carbohydrates, exude this mucilage constantly. The thickness of the polysaccharide capsule varies according to the phase of growth and cultivation conditions. During cell growth in batch cultures, aliquots of the polysaccharidic material of both capsules and colloidal mucilage are released into the surrounding medium, causing a progressive increase in its viscosity (De Philippis and Vincenzini, 1998). Apart from sugars, proteins and lipids are also exuded into the medium during cyanobacterial cultivation (Ishida *et al.*, 1997). Moreover, production of volatile hydrocarbons, halocarbons, and aldehydes has been observed in cyanobacterial cultures (Shaw, Chisholm, and Prinn, 2003; Muñoz, Mudge, and Sandoval, 2004).

The innumerable compounds formed during carbon biotransformation make it difficult to establish the complete carbon footprint in such systems. Therefore, further studies are necessary to determine the biological carbon-fixation routes qualitatively and quantitatively, with an aim to ascertain carbon mass balance in the photobioreactor and the environmental feasibility of these systems.

Table 4.1 Resource and development issues in cyanobacteria in carbon sequestration.

R&D issues in cyanobacteria in carbon sequestration	Comment
Cyanobacterial strains	How to select and maintain cyanobacterial strains that are competitive in open or closed photobioreactors is a central R&D issue in this field. Currently, only *Spirulina* strains have demonstrated this ability.
Genetics and molecular biology	After selection of the strains that can be used, these will need to be further improved by the application of molecular biology tools.
Physiology	In industrial conditions, cyanobacterial cultures are exposed to highly variable and often extreme environments. The knowledge of how cyanobacteria respond to stressors requires a deep understanding of their physiology.
Culture stability	Improving culture stability is a R&D challenge because cyanobacterial cultures are very susceptible to external contamination.
Process performance	Maximizing the performance of the process is the most important R&D objective in this field. Carbon dioxide removal efficiency and elimination capacity should be considered and a trade-off, generally determined by economic considerations, must be established between both parameters in the selection of particular processes.
Harvesting	Concentration of dilute suspensions of cyanobacteria has been an important issue in the downstream processing step.
Co-products, co-processes and biorefineries	The commercialization of carbon credits is not enough to justify cyanobacteria processes economically. The biorefinery concept should be introduced in order to enable the commercial exploration of this technology.
Bioreactor designs	New open and closed photobioreactor designs should be developed in order to balance the biological, engineering and economic aspects.
Environmental impact	Ultimately, the decision to apply cyanobacterial technologies for carbon dioxide mitigation will be made not only based on economic reasons but also taking into consideration their potential environmental impacts, i.e., how cyanobacteria-based processes can contribute to industrial sustainability.

4.7 Applicability of the process: the economics of cyanobacterial carbon sequestration

Raceway ponds and tubular photobioreactors are commercially the most frequently used bioreactor systems. These systems are economically feasible only when high-value products are involved in the production process. For carbon dioxide sequestration both reactors have poor performance, the conversion rates are low and therefore are not viable for processes aimed only to earn carbon credits. The intensification of the processes will be an important step in consolidating the technology for the biological transformation of carbon dioxide (using cyanobacteria) into photosynthetic products. Table 4.1 lists major hurdles that must be overcome before these processes can be fully scalable.

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Chapter 5

Ecology of cyanobacteria on stone monuments, biodeterioration, and the conservation of cultural heritage

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5.1 Introduction

Throughout history the most beautiful and durable stones have been used for the construction of buildings, which have become cultural treasures and part of our cultural heritage. Public art, historic buildings, and cultural heritage sites are a testimony to their creators, past cultures, and historic events. Microbial colonization on the exposed surfaces of monuments is responsible for their biodeterioration and represents a major international problem. Monuments and works of art in stone are made from different materials: sandstone, granite, limestone, dolerite, basalt, and also brick, plaster, mortar, and other building materials. Exposed to the open air over centuries, these materials deteriorate due to natural causes. While the weathering of rock to soil is an essential process for the evolution of life on Earth, biodeterioration of stone monuments is considered an unacceptable and irretrievable loss of our heritage and history. Open-air monuments, especially in the Mediterranean, and arid and semi-arid regions, including tropical areas, are exposed to high temperature, intense solar irradiation and drought for prolonged periods. The problems of biodeterioration are more serious in tropical regions with their hot and humid climate than in temperate areas, and hence

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there is a need to develop biological, chemical, and physical agents and approaches to protect our cultural heritage. Biofilms growing on the exposed surfaces of monuments have been intensively studied during recent decades, since there is a growing awareness of the importance of the preservation of cultural heritage (Gaylarde and Morton 1999; Ramirez *et al*., 2010).

5.2 Ecology of cyanobacteria growing on stone monuments

Microorganisms growing on stone monuments form a complex ecosystem, and they differ in their composition and function depending on the environmental conditions and on the physicochemical properties of the substratum.

Microbial colonization of stone monuments begins with phototropic microorganisms that produce a protective biofilm that is enriched with organic matter and with inorganic salts. Colonization by cyanobacteria as well as by other microorganisms accelerates the weathering of the substratum, and is followed by the appearance of heterotrophic bacteria, protozoa, and fungi that are attracted by the availability of organic matter and nutrients. This leads to the formation of complex microbial biofilms (Figure 5.1; Gaylarde and Marton, 1999). Although cyanobacteria and algal primary production are the source of carbon and nitrogen for the growth of heterotrophic bacteria and fungi, the re-mineralization by bacteria and fungi favors the growth of the phototrophs by providing carbon dioxide and nutrients. The biofilm grows on the surface of stone monuments but may also penetrate through pores in the stone up to a few millimeters.

Microbes growing on the surface of rock are known as epilithic while those inside the rock are called

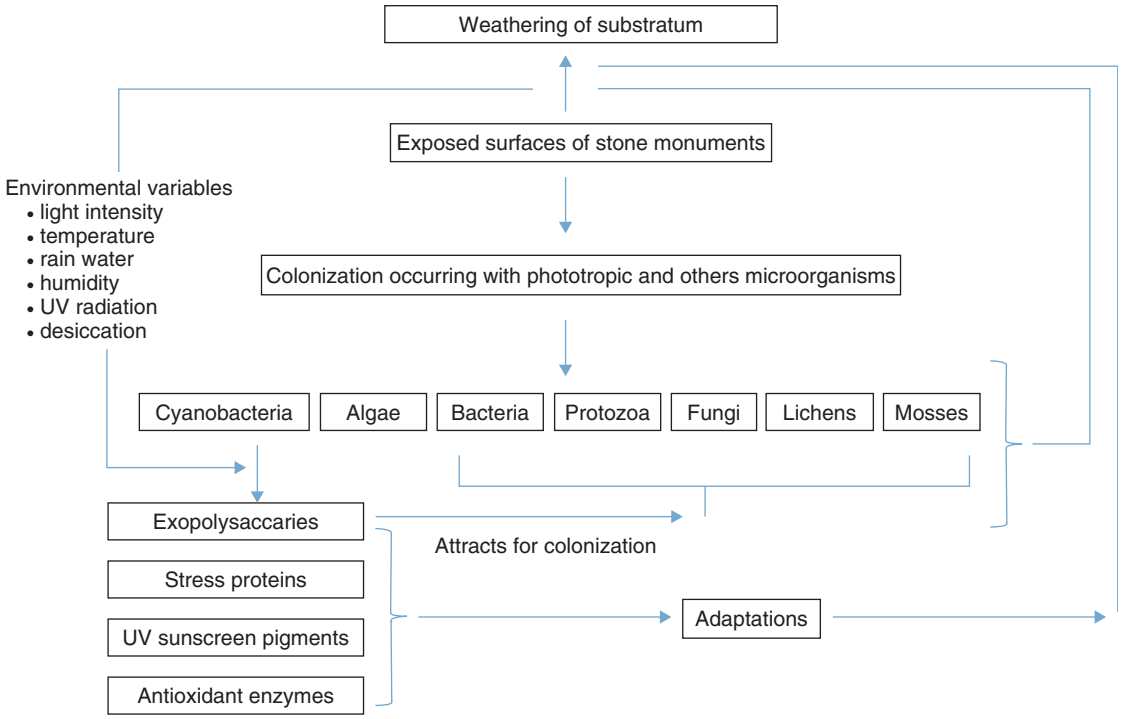

Figure 5.1 Microorganisms and phototropic organisms on the exposed surfaces of stone monuments and their mechanism of adaptation to environmental variables.

endolithic. Endolithic microbes are categorized into two types: chasmoendolithic microbes live in fractures in the stone that are connected to the surface while cryptoendolithic ones live inside the pores without a direct connection with the surface. Endolithic cyanobacteria are the major deteriorating organisms on the light-exposed surfaces of stone monuments (Videla, Guiamet, and Gomez de Saravia, 2000; Tomaselli *et al.*, 2000; Barberousse *et al.*, 2007). These cyanobacteria exude carbohydrates and growth factors, which provide a suitable environment for potentially more damaging organisms such as fungi, mosses, and higher plants (Grant, 1982; Gómez-Alarcón, Munõz, and Flores, 1994). Cyanobacteria mainly occur in microhabitats with high moisture content such as holes and crevices, depending on the type of substratum and the chemical and physical properties (Gaylarde and Gaylarde, 2000; Crispim, Gaylarde, and Gaylarde, 2003). In general, the black color of stone monuments is usually caused by the growth of cyanobacteria and other microorganisms that lead to the formation of biofilms or crusts of variable thickness (Pattanaik and Adhikary, 2002; Saarela *et al.*, 2004; Samad and Adhikary, 2008). This process is initiated mainly by the settlement of spores and airborne cells from the atmosphere, where they occur together with dust particles, known as aeroplankton. The growth of these organisms is affected by environmental conditions and the physicochemical properties of the substratum. Successful establishment of the deposited spores or cells mainly depends on their ability to attach rapidly and adhere strongly to the exposed surface. Their ability to adhere to these natural and artificial surfaces is mediated by extracellular polymeric substances (EPS), which are exuded by cyanobacteria under stress conditions such as desiccation and UV irradiation (Bertocchi *et al.*, 1990; Adhikary 1998; Rossi *et al.*, 2012). The thick biofilms provide shelter to the organisms against desiccation, UV radiation and high solar irradiation (Warscheid, Becker, and Resend, 1996). Biodeterioration occurs in different ways: the rock surface may be pitted, sandstone or granite may flake off in fairly large and flat pieces, glazes on brick surfaces may crack, and cryptoendolithic growth of microorganisms in sandstone can result in the formation of blisters. (Hirscha, Eckharde, and Palmer, 1995). The metabolic activity of biofilm organisms, such as the production of extracellular polymers, liberation of chelating compounds and/or organic/inorganic acids, together with the presence of pigments and the mechanical pressure exerted by growing structures, induce different types of damage:

- physical (abrasion, mechanical stress)
- chemical (dissolution, new-reaction products)
- aesthetic (colored patches or patinas and crusts).

The consequences of biological activity on stone surfaces include the formation of biofilms, colored patinas, encrustations, and the presence of vegetative and reproductive bodies. Moreover, these structures lead to dwindling, erosion, pitting, ion transfer, and leaching processes. In addition, the presence of extensive sheets of cyanobacteria trap water and retard desiccation, which exacerbates water-induced damage of the underlying substrate.

5.3 Factors affecting the occurrence of cyanobacterial biofilms on stone monuments and their biodeterioration activity

Biodeterioration can be defined as the breakdown of material by micro- or macro-organisms that colonize various objects, leading to irreversible loss of value and/or information. These organisms occur on the exposed surfaces of stone monuments under various environmental extremes due to their efficient adaptation mechanisms, and their presence leads to biodeterioration of the substratum (Figure 5.1). The interaction between substrate and microbes is known as bioreceptivity. Bioreceptivity can be defined as the susceptibility of the substrate to colonization, as well as describing the interactions between organisms and their products with the stone.

Colonization of microorganisms on stone monuments and biodeterioration are usually linked with the environment. The alteration and weathering of stones are mainly influenced by natural and anthropogenic

impacts on their physics, chemistry, and biology. The environmental conditions on the exposed stone surface or inside the pore space are extreme, since they are characterized by low water activity, low nutrient supply, large temperature fluctuations and high light intensities. When exposed outdoors, microorganisms do not so easily colonize polished surfaces, such as marble and compact stone. These surfaces first need to be weathered through environmental factors such as wind and atmospheric acids. Scaling is a phenomenon observed in decayed marble due to the inter-crystalline growth of microorganisms and is produced by different organisms such as fungi and bacteria (including cyanobacteria) (Gorbushina *et al.*, 1993; Tomaselli *et al.*, 2000). Stones exposed to solar radiation and located in extreme climates usually do not allow growth of many microorganisms on their surfaces, instead pushing them to a life below the surface where they are more protected. Patina (multilayered crust) formation is the consequence of the addition of exogenous material to the original surfaces, and both biological and inorganic factors are involved (Garcia-Valles *et al.*, 2000). Factors affecting biofilms on stone monuments and their biodegradation are:

- type of stones and nature of their surfaces
- enrichment of inorganic and organic nutrients
- water availability determined by material-specific parameters such as porosity and permeability as well as environmental conditions
- biomass accumulated by the activity of photosynthetic microorganisms (Palmer and Hirsch, 1991)
- anthropogenic pollutants.

The mechanisms of protection of biofilm organisms against harmful environmental conditions include the enrichment of pigments and the exudation of EPS (Gaylarde and Morton, 1999). EPS acts as a coating that can change the physicochemical surface properties by increasing water binding capacity as well as decreasing surface roughness. Extracellular exudations show a high affinity for bivalent metal cations, and hence bind to the minerals of stone substrata and weaken them (Rossi *et al.*, 2012). Light intensity and humidity (Ortega-Calvo *et al.*, 1995) are important factors that determine the composition of phototrophic microbial communities in biofilms.

5.4 Geographical distribution of cyanobacteria on rock and stone monuments

Cyanobacteria are the dominant photosynthetic microorganisms on stone monuments. They form conspicuous biofilms at the interface of a solid substratum and the atmosphere in all climatic zones (Ortega-Calvo *et al.*, 1995; Tomaselli *et al.*, 2000). Sub-aerial cyanobacteria forming brownish-black crusts or biofilms on stone monuments have been reported from almost all climatic zones. Examples come from India (Tripathy *et al.*, 1997, 1999; Pattanaik and Adhikary, 2002; Samad and Adhikary, 2008; Khan and Kulathuran, 2010), France (Crispim, Gaylarde, and Gaylarde 2003), Greece (Anagnostidis *et al.*, 1983), Italy (Tomaselli *et al.*, 2000; Lamenti, Tiano, and Tomaselli, 2000; Bellinzoni, Caneva, and Ricci, 2003), Portugal (Oliveira *et al.*, 2011), Slovakia (Uher and Kováčik, 2004; Uher *et al.*, 2005; Uher, 2008) and Spain (Saiz-Jimenez et al., 1991; Ortega-Calvo *et al.*, 1993; Gómez-Alarcón *et al.*, 1995; Zurita *et al.*, 2005; Uher *et al.*, 2005). Sub-aerial bacteria are particularly abundant in the tropics and much less so in temperate regions. These unicellular and filamentous cyanobacteria belong to the orders Chroococcales, Oscillatoriales, Nostocales, and Stigonematales (Table 5.1). The geographical distribution of sub-aerial cyanobacteria on stone monuments with respect to different climatic regimes of the globe are summarized in Table 5.1 and are depicted in Figure 5.2. A representative photograph of a stone/mortar monument in Santiniketan, West Bengal, in eastern India showing blackish coloration of the surfaces due to colonization of cyanobacteria is given in Figure 5.3.

Although aero-terrestrial cyanobacteria are important from an ecological point of view, their eco-physiology in relation to desiccation has not been thoroughly investigated. Only a few species of cyanobacteria, for example *Nostoc commune*, *Chroococcidiopsis* sp., *Anabaena* sp., *Nostoc flagelliforme*, *Tolypothrix byssoidea*, *Chroococcus turgidus*, *Lyngbya arboricola*, *Microcoleus* sp., *Nostoc punctiforme*, *Phormidium autumnale*, *Stigonema crassum*,

Table 5.1 Occurrence of cyanobacteria on the exposed surfaces of stone monuments by climatic region.

Order/genera	Tropical region			Temperate region					
	India	Brazil	Portugal	Spain	France	Italy	Slovakia	Greece	Korea
Chroococcales									
Cyanobacterium	−	−	−	+	−	−	−	−	−
Aphanothece	−	−	−	+	+	−	+	−	−
Gloeothece	+	+	−	−	−	−	−	−	−
Synecococcus	−	+	+	+	−	+	−	+	+
Synechocystis	−	+	+	+	−	+	−	−	+
Aphanocapsa	−	−	−	+	+	+	−	+	+
Merismopedia	−	−	−	+	−	−	−	−	−
Gomphosphaeria	−	−	−	+	−	−	−	−	−
Gleocapsa	+	+	−	+	+	+	−	−	−
Eucapsis	−	−	−	−	−	+	−	−	−
Gloeocapsopsis	+	−	−	−	−	−	−	−	−
Chrocoococcus	−	+	−	+	−	+	+	+	+
Cyanosarcina	−	−	−	−	−	−	−	+	−
Pseudocapsa	−	−	−	+	−	−	−	−	−
Chlorogloea	−	−	−	+	−	+	−	−	−
Hydrococcous	−	−	−	−	−	−	−	−	−
Stichococcus	−	−	−	−	−	+	−	−	−
Chamaesiphon	−	−	−	+	−	−	−	−	−
Dermocarpa	−	−	−	−	−	−	−	+	−
Chroococcidiopsis	+	+	+	−	−	+	+	−	−
Myxosarcina	+	+	−	−	−	+	−	−	−
Xenococcous	−	+	−	−	−	−	−	−	+
Pleurocapsa	−	+	−	+	−	+	−	−	−
Hyella	−	−	−	+	−	+	−	−	−
Oscillatoriales									
Pseudoanabaena	−	−	−	+	−	−	−	−	−
Geitlerinema	−	−	−	−	−	+	−	−	−
Leptolyngbya	+	+	−	+	−	+	+	+	−
Heteroleibleinia	−	−	−	−	−	−	+	−	−
Schizothrix	−	−	−	+	+	−	−	+	−
Borzia	−	−	−	−	−	−	−	−	+
Pseudophormidium	−	−	−	−	−	−	−	−	−
Phormidium	+	+	+	+	−	+	+	+	−
Symploca	−	−	−	+	−	−	−	−	−
Microcoleus	+	−	−	+	−	+	+	+	−
Hydrocoleum	−	−	−	−	−	−	−	+	−
Oscillatoria	+	+	−	−	+	−	−	−	+

(*continued overleaf*)

Table 5.1 (*continued*)

Order/genera	Tropical region			Temperate region					
	India	Brazil	Portugal	Spain	France	Italy	Slovakia	Greece	Korea
Lyngbya	+	+	−	−	−	+	−	−	+
Plectonema	+	−	+	+	−	−	−	+	−
Nostocales									
Cylindrospermum	−	−	−	+	−	−	−	−	−
Nostoc	+	−	−	+	−	+	+	+	−
Anabaena	−	−	−	−	−	−	−	−	+
Scytonema	+	+	−	+	−	+	−	+	+
Tolypothrix	+	+	−	+	−	+	−	−	−
Calothrix	+	+	−	+	−	+	−	−	−
Stigonematales									
Hapalosiphon	+	−	−		−	−	−	−	+
Westiellopsis	+	−	−	−	−	−	−	−	−
Fischerella	+	−	−	−	−	+	−	−	−
Stigonema	+	+	−	−	+	−	−	−	+

(+) indicates the presence of the cyanobacterium; (−) the absence

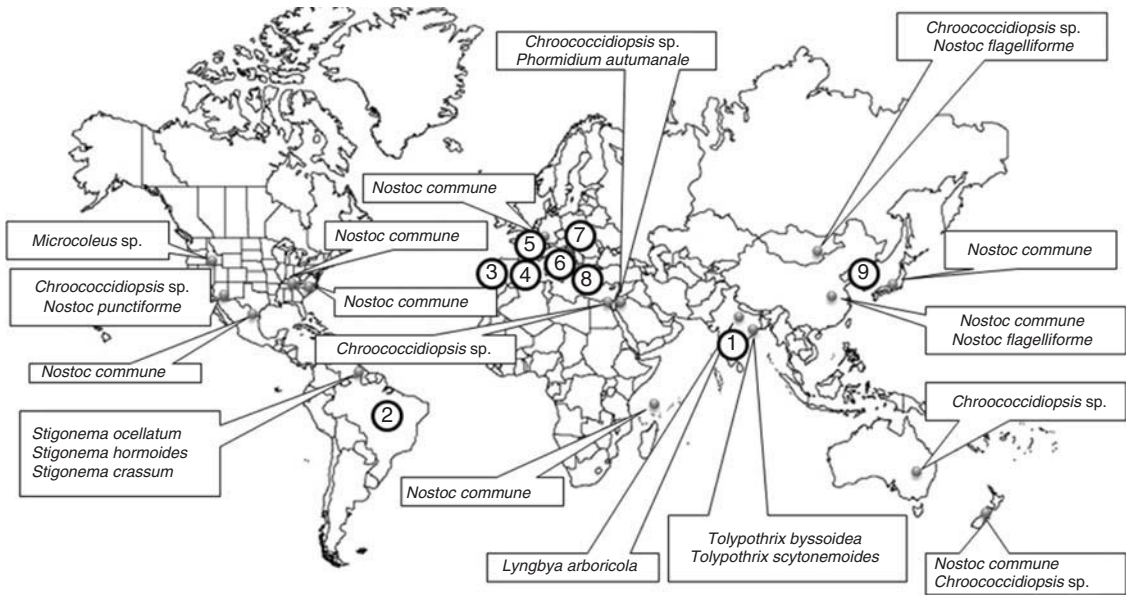

Figure 5.2 World map showing the places in different countries from where cyanobacteria species on stone monuments have been reported (1–9); the small close circles show the locations where desiccation-tolerant cyanobacterial species have been isolated from different types of habitats and used in experiments by various scientific groups.

Figure 5.3 View of the exterior of stone/mortar monuments at Santiniketan, West Bengal showing colonization of phototropic biofilms imparting blackish coloration to the substratum. See plate section for color version.

Stigonema hormoides, *Stigonema ocellatum*, and *Tolypothrix scytonemoides* have been investigated with respect to anhydrobiosis and most of the experiments were carried out with *N. commune* and *Chroococcidiopsis* sp. (Figure 5.4).

5.5 Taxonomy and biodiversity of cyanobacteria on stone monuments

Biodiversity assessment of microbial communities on exposed stone surfaces of monuments has mainly been approached by following traditional morphometric analyses. Most cyanobacteria exhibit defined morphological characters and can be identified through light-microscopic observation. However, it has often proven difficult to identify them solely on this basis. Cyanobacteria may show a high degree of polymorphism and they may change their structure as the result of adaptation to extreme anhydrobiosis of the terrestrial habitats. Hence it is thought that only a few cyanobacteria from stone monuments have been correctly identified. Studies based on 16S ribosomal RNA gene sequencing have demonstrated that a much higher number of cyanobacteria inhabit these biofilms (Gaylarde *et al.*, 2004). Biodiversity analysis and proper identification of biofilm cyanobacteria and other microorganisms is now based on a polyphasic approach, in other words using a combination of morphological, physiological, biochemical, and molecular-genetic approaches. This is necessary because in most restoration work conventional biocides are applied, and these are probably not suitable for the unknown and complex microbial communities growing on monuments. Morphological identification by light microscope also has its limitations because spores, akinetes, and hormogonia cannot be identified unless they are cultured, which often takes a long time or may even be impossible for anhydrobiotic cyanobacteria. These cyanobacteria often show large morphological variations so that even a trained taxonomist often fails to identify them correctly. To overcome these drawbacks, the use of molecular-genetic techniques is highly recommended. These techniques can be broadly grouped as:

- PCR-independent approaches, including guanine plus cytosine estimation, nucleic acid re-association and hybridization, and DNA microarrays.
- PCR-based approaches, including denaturing gradient gel electrophoresis, temperature gradient gel electrophoresis, restriction fragment length polymorphism, single strand conformational polymorphism, ribosomal intergenic spacer analysis, and randomly amplified polymorphic DNA.

For the molecular assessment of cyanobacterial biodiversity markers such as the 16S rRNA gene, the ITS region (the spacer region located between the 16S and 23S rRNA genes), the phycocyanin locus, *nif* genes, *rpo* gene, and others are frequently used. The increasing interest in the ecology of cyanobacteria and other microorganisms colonizing monuments has lead researchers to use the polyphasic approach, described

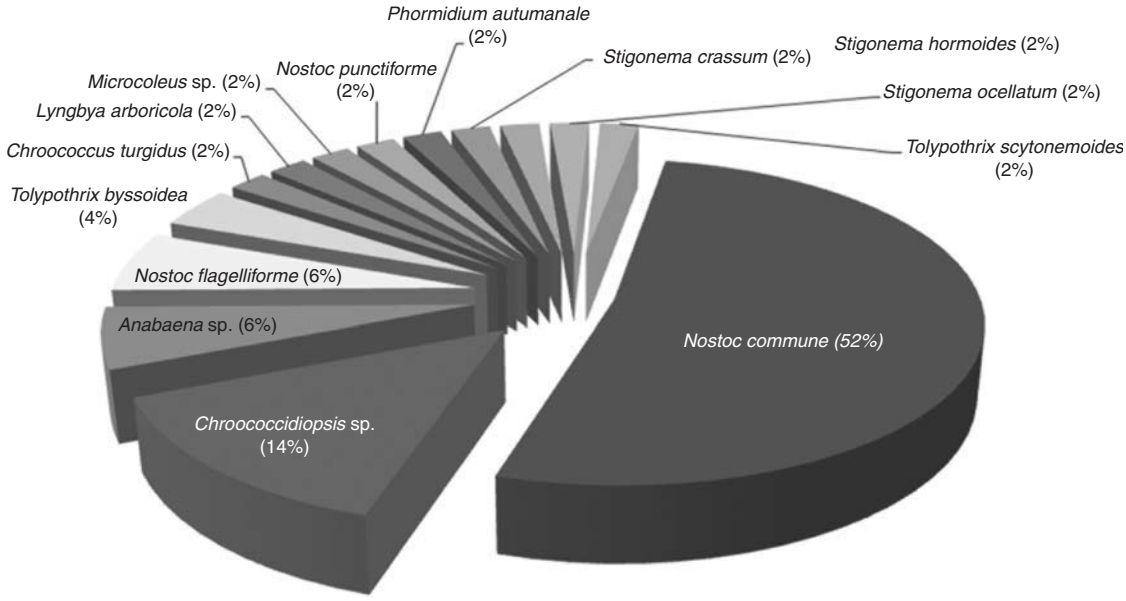

Figure 5.4 Percentage of desiccation-related work so far carried out on different cyanobacteria species.

above, in order to better understand the process of colonization and weathering of the cultural heritage.

5.6 Stress-tolerance mechanisms in terrestrial cyanobacteria

Cyanobacteria in terrestrial habitats are subject to a variety of stresses, such as high light intensities, ultraviolet radiation, high and low temperatures, osmotic stress, salinity, drought, and desiccation (Allakhverdiev *et al.*, 2000; Singh, Sinha, and Häder, 2002; Lin *et al.*, 2004; Potts *et al.*, 2005; Büdel *et al.*, 2008). Compared to freshwater and marine environments, aero-terrestrial cyanobacteria in the tropics are exposed to more adverse environmental conditions, especially desiccation and high solar irradiation and UV. The resistance of these cyanobacteria to extreme environmental conditions provides them with a distinct advantage for survival on exposed surfaces.

Endowed with effective protective or defense mechanisms against various abiotic stresses, and a remarkable adaptability to varying environmental conditions, aero-terrestrial cyanobacteria successfully colonize and inhabit almost any type of terrestrial habitat, including rocks and the external walls of monuments or building facades, and are known as lithobiontic (rock-inhabiting) or epilithic (rock-surface-inhabiting) organisms. Although these organisms are inactive when desiccated, they can recover quickly when water becomes available, which shows their desiccation tolerance (Scherer and Potts, 1989; Higo *et al.*, 2007). The terrestrial cyanobacterium *Nostoc commune* retains viability for over 100 years when desiccated (Lipman, 1941; Cameron, 1962). Even when these cyanobacteria are subject to repeated cycles of dehydration, desiccation, and rehydration, they survive under desiccated conditions for prolonged periods of time on the exposed surfaces of stone monuments. Thus anhydrobiotic cyanobacteria are responsible for the biodeterioration of stone monuments mainly because they can cope with the adverse environmental conditions found there.

Stress tolerance is the sum of many alterations of the organisms at the structural, physiological, and molecular levels, due to their adaptation to a changed environment. The formation of a sheath or mucilage envelope and/or the development of akinetes are the main morphological alterations at the structural level.

Crowding of cytoplasmic components, condensation of the nucleoid, and an increase in the melting temperature (T_m) of membrane phase transitions occur in cyanobacteria that have been subjected to desiccation (Dadheech, 2010). Cyanobacteria inhabiting the exposed surface of stone monuments and subjected to intense solar irradiation produce reactive oxygen species leading to desiccation damage (Adhikary, 2008). *Nostoc commune* retains its ultra-structural organization and the integrity of its intracellular membranes even after two years of desiccation (Peat, Powell, and Potts, 1988). Upon being subjected to desiccation the envelope of the cyanobacterium *Chroococcidiopsis* sp. shows the presence of sporopollenin-like compounds, sulfated- and beta-linked polysaccharides, positively charged glycoproteins, lipids, and proteins (Grilli-Caiola, Billi, and Friedmann, 1996). Loss of water from cells is accompanied by the destruction of macromolecules such as proteins and nucleotides, as well as changes in the lipoprotein arrangement of membranes (Dadheech, 2010). Upon desiccation, Maillard reactions and metal-dependent Fenton reactions damage proteins, nucleic acids, and membrane components (Dadheech, 2010). However, survival during water stress is achieved by the regulation of ion fluxes for immediate osmotic adjustment to prevent turgor pressure loss, selective expression of tolerance genes and stress proteins, scavenging of reactive oxygen species by oxygen-scavenging enzymes (e.g., superoxide dismutases, ascorbate peroxidase, and catalases), downregulation of metabolism, and accumulation of certain amphiphilic solutes, proteins, and polysaccharides (Oliver *et al.*, 2001). Cyanobacterial crusts collected from the hot terraces of Varanasi, India, during the summer months (60–65°C) were intensely stained after treatment with 2, 3, 5, triphenyl-tetrazoliun chloride, indicating the presence of a high level of SH-groups in the cells (Tripathy and Talpasayi, 1980). Desiccation-tolerant photosynthetic organisms possess mechanism(s) to avoid photoinhibition under conditions of desiccation, as well as a mechanism(s) to cope with desiccation itself.

5.6.1 Nucleic acids

Polynucleotides of cyanobacteria such as ribosomal RNA have been found to be remarkably stable in a state of desiccation maintained for many years. 16S rRNA in particular is more stable in the desiccation-tolerant *N. commune* than in planktonic species of the Nostocaceae (Han and Hu, 2007).

The synthesis of DdrA plays a key role in recovery from DNA destruction caused by radiation as well as desiccation. DdrA is a protein with an affinity for the ends of single-stranded DNA that protects from nuclease digestion. Among cyanobacteria, the genome of *Gloeobacter violaceus* isolated from exposed limestones in the Swiss Alps contains the gene *glr*0712, the product of which showed a similarity of 40% with the DdrA protein (Potts *et al.*, 2005). The nitrogen-fixing filamentous cyanobacteria *Anabaena* sp. PCC7120 showed an inherent capacity for desiccation tolerance. In this organism the genes coding for osmoprotectants and the K^+ transporting system are upregulated from early- to mid-desiccation. In the same cyanobacterium, Katoh, Asthana, and Ohmori (2004) have reported that the heat-shock proteins were upregulated after mid-desiccation, while photosynthesis and nitrogen-transport systems were downregulated during early desiccation. RNA polymerase and ribosomal proteins were downregulated between the early and the middle phase of desiccation and the genes induced by osmotic, salt, and low-temperature stress were upregulated under desiccation. Higo *et al.* (2007) have shown that in *Anabaena* sp. PCC 7120 expression of genes was involved in DNA repair, protein folding, nitrogen depletion and carbon dioxide limitation during rehydration, although this was not the case during dehydration. Two genes, *ancrpB* and *alr0618*, encode putative transcriptional activators of the cAMP receptor protein family, while AnCrpB and Alr0618 regulate the genes induced by nitrogen depletion and by carbon dioxide limitation, respectively. Thus, during rehydration, the expression of these genes responsible for specific metabolism of the cyanobacterium was induced (Higo *et al.*, 2007).

5.6.2 Proteins

Proteins remain stable despite extended periods of desiccation. Heat-shock proteins were detected in the terrestrial epilithic cyanobacterium *Tolypothrix byssoidea* (Adhikary, 2003). The desiccation-tolerant cyanobacterium *N. commune* accumulates a novel

group of acidic proteins of isoelectric points between 4.3 and 4.8 and apparent molecular masses between 30 and 39 kDa, a water-stress protein with a protective function (Scherer and Potts 1989; Hill *et al.*, 1994a, b; Potts *et al.*, 2005). These proteins are stable for decades. Twelve major proteins were detected in the cytoplasmic membrane of *Nostoc commune* UTEX 584, and the most abundant of these proteins had molecular masses of 83, 71, 68, 51, and 46 kDa (Olie and Potts, 1986). Two unidentified glycoproteins (75 kDa and 110 kDa), an unidentified 68 kDa protein, the second most abundant protein in aqueous extracts of the glycan in *Nostoc commune* were also detected (Hill *et al.*, 1994a). Acidic water-stress polypeptides (Wsp) with molecular masses of 33, 37, and 39 kDa were the most abundant soluble proteins in *N. commune* and showed homology with the sequences of carbohydrate-modifying enzymes associated with a 1, 4-β-D-xylanxylanohydrolase activity (Hill *et al.*, 1994b). Transcription of *wspA* and *sodF* (superoxide dismutase), and synthesis and secretion of WspA (33.6 kDa) were induced upon desiccation or UVA/B irradiation of the cells. WspA, which accounted for more than 70% of the total soluble protein, plays a central role in mitigating water stress in organisms (Wright *et al.*, 2005). Serine, glycine, proline, lysine, and cysteine occurred in higher concentrations in cells of the terrestrial cyanobacterium *Tolypothrix sytonemoides* subjected to desiccation than in cells not subjected to desiccation (Rajendran, Kathirvel, and Anand, 2007).

5.6.3 Polysaccharides

The major compatible solutes found in desiccation-tolerant cyanobacteria are sugars, such as sucrose and particularly trehalose (Hershkovitz Oren, and Cohen, 1991; Potts, 1994; Singh, Sinha, and Häder, 2002; Hincha and Hagemann, 2004; Sakamoto *et al.*, 2009), sugar derivatives such as 2-O-glucopyranosyl-glycerol, or betains such as glycine-betain and glutamate-betain (Allakhverdiev *et al.*, 2001; Hincha and Hagemann, 2004). Compatible solutes stabilize macromolecules and membranes by means of hydroxyl groups, for example in sugars and sugar alcohols, allowing the formation of hydrogen bonds, and by buried electrical charges such as the positive charge of the quaternary nitrogen in various betains. Desiccation-tolerant cyanobacteria are able to survive nearly complete loss of water through the production of internal and external polysaccharides (Crowe, Crowe, and Chapman, 1984; Carpenter, Crowe, and Crowe, 1987). Trehalose has been reported to play a central function in regulating water uptake, protecting protein and membrane structures under dry conditions (Grilli-Caiola, Ocampo-Friedmann, and Friedman, 1993, 1996; Leslie *et al.*, 1995). In dehydrated *Anabaena* cells, the expression of *treZ*, *treY*, and *treH* genes are involved in the synthesis of trehalose (a-D-glucopyranosyl- [1, 1] -a-D-glucopyranoside) and synthesized by the TreY–TreZ pathway from a (1, 4)-linked glucose polymer (Katoh, Asthana, and Ohmori, 2004; Higo *et al.*, 2006; Yoshida and Sakamoto, 2009). Maltooligosyl trehalose synthase, encoded by the *treY* gene, converts the terminal (1, 4)-linked residue of the glucose polymer to a (1, 1) linkage. Maltooligosyl trehalose trehalohydrolase, encoded by the *treZ* gene, then produces free trehalose by cleavage of the terminal disaccharide (De Smet *et al.*, 2000). Trehalose is indirectly related to desiccation tolerance by inducing the gene encoding a cofactor of the chaperone DnaK (Higo *et al.*, 2006). Sucrose and trehalose increase the stability of native proteins and assist in refolding of unfolded polypeptides, and hence they serve as chemical chaperones (Hottinger *et al.*, 1994, Singer and Lindquist 1998). These polysaccharides stabilize the cells during prolonged storage in the air-dried state, at low or high temperatures (Higo *et al.*, 2006). First, the glycan inhibits fusion of membrane vesicles during desiccation and freeze-drying, and acts as an immobilization matrix for a range of secreted enzymes, which remain fully active after long-term air-dried storage (Hill *et al.*, 1994b). Second, the glycan provides a structural and/or molecular scaffold with rheological properties, which can accommodate the rapid biophysical and physiological changes in the community upon rehydration and during recovery from desiccation (Shirkey *et al.*, 2000). The glycan swells from a brittle dried crust to a cartilaginous structure within minutes of rehydration. Third, the glycan matrix contains both lipid- and water-soluble UV-radiation-absorbing pigments, which protect the cell from photo-degradation (Scherer and Potts 1989). Fourth, although epiphytes

colonize the surfaces of *Nostoc* colonies, there is no penetration of the glycan due in part to a silicon- and calcium-rich pellicle and inherent resistance of the glycan to enzymatic breakdown (Scherer and Potts 1989).

Extracellular polysaccharides (EPS) play a major role in protecting the cells of cyanobacteria from various stresses in extreme habitats (Bertocchi *et al.*, 1990; Gloaguen, Morvan, and Hoffmann, 1995). Hydrogen bonds with proteins, lipids, and DNA, essentially replacing the water shell around the molecules, are probably created by the polysaccharides secreted by cyanobacteria under desiccation stress (Potts, 1994). Preliminary structural work on one water-soluble UV-absorbing pigment (released from the glycan by acid hydrolysis) indicates the presence of an oligosaccharide (Böhm *et al.*, 1995), raising the possibility that the pigment may be covalently linked to the glycan in the desiccated state.

5.6.4 Lipids

Lipids are particularly important as they form the phospholipid bilayers of cellular membranes. The preservation of the structural integrity of membranes is highly important for processes that are active during dehydration, desiccation, and rehydration, and to control the many membrane functions such as transport processes and the energy-conservation metabolism. Hydrogen atoms of the olefinic bonds are highly susceptible to oxidative attack. It appears that unsaturation of the fatty acids of lipids counteracts any stress effects (Gombos *et al.*, 1997). For photosynthesis of desiccation-tolerant cyanobacteria, it is interesting to note that with unsaturation the photosynthetic machinery in particular is protected. The repair of damaged photosystems I and II (PS I, PS II) is enhanced (Gombos, Wada, and Murata, 1994; Allakhverdiev *et al.*, 2001; Singh, Sinha, and Häder, 2002) and the important turnover of the D1 protein of PS II is sustained. However, it appears that these effects are not directly the result of changes in the photosynthetic machinery in the membranes, but are caused more indirectly by the cytoplasmic environment, which is controlled by water and ion transport through the cytoplasmic membrane (Allakhverdiev *et al.*, 2001). The content of palmitic acid, palmitoleic acid, heptadecanoic acid, linoleic acid, and myristic acid is elevated in desiccated cells of *Tolypothrix sytonemoides* in comparison to its hydrated cells. Moreover, myristoleic acid, eicosenoic acid, and behenic acid are synthesized by desiccated cells of the organisms while tricosanoic acid is synthesized by its hydrated cells (Rajendran, Kathirvel, and Anand, 2007). These findings show that the quantity of certain fatty acids is higher in desiccated cells of cyanobacteria, which may be responsible for its survival under desiccation.

5.6.5 UV-absorbing compounds and pigments

Most cyanobacteria that inhabit stone monuments and other terrestrial environments are exposed to direct or indirect solar irradiation along with potentially damaging levels of ultraviolet radiation (UVR). For many terrestrial cyanobacteria (e.g., in desert crusts, intertidal mats, and endolithic environments), life in exposed areas can also make them susceptible to desiccation. Hence these organisms must have developed strategies to cope with UVR as well as desiccation stress. The synthesis of carotenoids and tocopherols protect the cells against oxidative damage caused by desiccation. Desiccation of cyanobacteria at sites in the field is naturally highly correlated with high solar irradiance. In extremely sun-exposed habitats, UV protection is provided by the production of effective sun-blocking pigments, such as mycosporine-like amino acids (MAAs) and the indol-alkaloid scytonemin (Garcia-Pichel and Castenholz, 1991; Büdel, Karsten, and Garcia-Pichel, 1997; Sinha *et al.*, 1998, 2001; Sinha, Klisch, and Häder, 1999). MAAs are small (<400 Da), colorless, water-soluble compounds composed of cyclohexenone or cyclohexenimine chromophores conjugated with the nitrogen substituent of an amino acid to its imino alcohol. Scytonemins (544 Da) are yellow-brown lipid-soluble pigments located in the extracellular polysaccharide sheath of cyanobacteria. The MAAs in *N. commune* CHEN/1986 form strong interactions with a collection of proteins called the water-stress proteins (Wsp) (Scherer and Potts, 1989), which are also present in substantial amounts in desiccated colonies (approximately 60% of the total soluble protein) and are released from colonies upon

rehydration (Hill *et al.*, 1994a). Scytonemin synthesis is predominantly induced by UVA radiation, but high-intensity violet/blue light may also induce synthesis of this pigment, and it is further affected by many other environmental factors. The role of scytonemin as an UVR screening compound for cyanobacteria in liquid culture is well established (Garcia-Pichel, Sherry, and Castenholz, 1992; Dillon and Castenholz, 1999). Scytonemin is possibly more important as an UVR screen during periods of desiccation, when damage could accrue while alternative UVR coping mechanisms are inactive: cells therefore accrue less UVR damage while desiccated and thus require less energy for the repair and recovery of cellular systems during rehydration. Purification and biochemical analysis of the cytoplasmic membrane from the desiccation-tolerant cyanobacterium *Nostoc commune* showed the presence of β-carotene and echinenone as the dominant carotenoids, with lesser amounts of zeaxanthin and (3R)-cryptoxanthin and a lack of chlorophyll *a* and pheophytin (Olie and Potts, 1986). β-carotene and echinenone predominate in axenic cultures of *N. commune* UTEX 584 and canthaxanthin, and the β–γ series of carotenoids in its desiccated field materials (Potts *et al.*, 1987). Scytonemin synthesis was increased in *Nostoc punctiforme* PCC 73102 and *Chroococcidiopsis* CCMEE 5056 but partly inhibited in *Chroococcidiopsis* CCMEE 246 upon periodic desiccation. Produced scytonemin is extremely stable and intact in the sheaths of desiccated *N. punctiforme* (Fleming and Castenholz, 2007).

5.6.6 Antioxidant enzymes

Mechanisms operating to protect cells from the effects of desiccation also circumvent oxidative damage. Cyanobacteria were among the first organisms to evolve effective mechanisms for protection from oxidative stress (Aro, Virgin, and Andersson, 1993; Mittler, 2002). The ROS-scavenging systems in cyanobacteria include enzymes such as superoxide dismutases, catalases, peroxidases, and peroxiredoxins and other antioxidant enzymes. The synthesis of superoxide dismutase (SOD) is a mechanism that acts against the damage caused by desiccation. SOD mediates the disproportionation of superoxide radicals to hydrogen peroxide and dioxygen; the hydrogen peroxide is then scavenged either by ascorbate peroxidase or catalase. SODs are ubiquitous metalloenzymes that exist in four forms depending upon the nature of their catalytic metals: FeSOD, MnSOD, Cu/ZnSOD, and NiSOD (Fridovich, 1997). Catalases exclusively dismutate hydrogen peroxide, whereas peroxidases use a broad range of peroxides (ROOH) as substrate (Chelikani, Fita, and Loewen, 2004). The extracellular polysaccharide (glycan) of *N. commune* DRH1 generates superoxide radicals upon exposure to UVA or UVB irradiation and these radicals are scavenged by SOD. Active Fe-SOD (SOD F) was the third most abundant soluble protein in cells of *Nostoc commune* CHEN/1986, and was detected even after 13 years of storage of the organism in a desiccated state. *SodF* encodes an open reading frame of 200 codons and is expressed as a monocistronic transcript (of approximately 750 bases) from a region of genome that includes genes involved in nucleic acid synthesis and repair, including dipyrimidine photolyase (*phr*) and cytidylate monophosphate kinase (*panC*) (Shirkey *et al.*, 2000). The high levels of *sodF* in *N. commune* and the enzyme released from cells upon rehydration counteract the effects of oxidative stress imposed by multiple cycles of desiccation and rehydration during UV irradiation *in situ*. The activity of SOD, ascorbate peroxidase, and catalase increases in *Lyngbya arboricola* with the length of the desiccation period (Tripathy and Srivastava, 2001). Five isoforms of SOD were detected: one Mn-SOD, one Fe-Mn-SOD, and three Fe-SOD. Ascorbate peroxidase occurs in two isoforms while catalase occurs in three in *Lyngbya arboricola* (Tripathy and Srivastava, 2001). Higher amounts of superoxide dismutase were detected in *Tolypothrix byssoidea* isolated from the exposed rock surface of a stone monument, the Sun Temple of Konark, in Odisha state, India. Only one form of SOD, Fe-SOD, was detected in this organism, as a 292-bp-size *sodB* gene (Sahu and Sabat, 2002; Adhikary and Klug, 2007). In *Tolypothrix scytonemoides* after desiccation for more than three months, the activities of catalase and superoxide dismutase were enhanced by 42.8% and 8.1% respectively (Rajendran, Kathirvel, and Anand, 2007). In *Nostoc flagelliforme*, SOD remained active even after three years of drying and its activity was 78% of that in fully recovered samples (Qiu, Zhang, and Liu, 2003).

5.7 Control methods of cyanobacteria on stone monuments

The weathering of stone monuments is not a recent phenomenon and it has been understood for a long time that it is caused by both biotic and abiotic factors. Lithobiontic cyanobacteria are potent promoters of deterioration, causing aesthetically unacceptable discoloration and biodeterioration of the colonized surfaces of the walls of monuments, temples, and other historical buildings. This tendency mainly depends on the availability of carbon and energy sources to sustain growth of microbes, the metabolic activities of which cause irreversible mineral biotransformation and biomineralization of organic materials on the substratum (Sánchez-Moral *et al.*, 2003, 2004). This results in the formation of biofilms on the exposed surfaces of monuments, with the microorganisms embedded in a matrix of exopolysaccharides (Albertano, Luongo, and Grilli-Caiola, 1991). These biofilms must be removed regularly, using non-abrasive and environmentally safe methods, or the impact of microbial activities on the surfaces they colonize must be minimized. The use of microscopic fluorescence techniques on the stone exteriors can detect the presence of phototropic communities, which are involved in biodeterioration due to their auto-fluorescence.

Biodeterioration of monuments and other parts of the cultural heritage by cyanobacteria and other microorganisms has been observed globally. But only a few attempts have been made to identify chemical agents suitable for the control of these organisms. Continuous attempts have been made to slow down the deterioration of monuments by the treatment with preservatives. The drawback of this approach is that these materials have different chemical compositions and thermal expansion coefficients to the stone. Moreover, these agents are usually formulated and applied in solvents at very low concentration. Hence a large quantity of organic solvent is released into the environment. The chemical nature of these solvents and the long periods for which they persist means that they are themselves also detrimental to the conservation of the stone. Thus, there is an urgent need for further research to discover chemicals that can be employed for the control and monitoring of phototropic biofilms on stone monuments. The chemicals used for conservation of monuments and building facades and the concentrations involved are:

- Polyvinyl acetate (1–2%) in toluene, Teepol (detergent) solution (Subbaraman, 1993).
- Polyvinyl acetate (1–2%) in ethanol or rectified spirit, benzene (Subbaraman, 1993).
- n-Butyl amine or cyclohexyl amine (5–10%) in a neutral solvent such as petroleum spirit (Subbaraman, 1993).
- Ammonium (2–5%) solution in alcohol and formalin or pentachlorophenol (2%) (Subbaraman, 1993).
- Alcoholic ammonia solution (5%), formalin or pentachlorophenol (2%) plus one part of acetone, one part of amyl acetate, four parts of diacetone alcohol, four parts of cellosolve, a few drops of morpholine, dimethyl formamide (Subbaraman, 1993).
- Mixture of triethanol amine and Teepol in rectified spirit (2%) in 1:1 proportion (Subbaraman, 1993).
- Instakill (10–2000 ppm) composed of a mixture of $FeSO_4$ and $Al_2(SO_4)_3$ in the ratio of 4:1 with gum Acacia (0.5%) as adhesive (Kaushik and Kumar, 1995).
- Potassium sorbate (2, 4 hexadienolic acid potassium salt), hyamine 1622 (diisobutyl-phenoxye thoxyethyl-dimethyl-benzyl-ammonium chloride) and BCI (alkyl-benzyl-diethyl-ammonium chloride) mixed with one of two types of glue:
  - Colleta (prepared by melting 10 g of cow glue and 10 g of rabbit glue in 200 ml of tap water).
  - Re-lining paste (prepared by melting 32 g of cow glue, 25 g of linen seeds, 30 g of rye flour, 30 g of wheat flour, and 16 g of turpentine in 200 ml tap water).

  The final concentration in the glue is 1–2% for each product (Tiano, Tosini, and Rizzi, 1997):
- $CuSO_4$ (0.01, 0.05, 0.1, 0.5, and 1% w/v); $ZnSiF_6$ (0.01, 0.1, 0.5, and 1% w/v); $Al_2(SO_4)_3$ (0.01, 0.5, 1, and 5% w/v) and ammonia (0.01, 0.05, 0.1, 0.5, and 1% w/v) (Adhikary, 2002).
- Synthetic polypeptide poly-aspartate (Poly A and Poly AF) in a concentration of 80 mg/l in $CaCl_2$ (7.5 mM) (Tiano, 2004).
- Monochromatic light (orange, green, blue) (Albertano, Bruno, and Bellezza, 2005).

5.8 Concluding remarks and future perspectives

Colonization of microorganisms on the exterior surface of stone monuments causing biodeterioration leads to the irretrievable loss of cultural property and history and represents a major international problem. It is more problematic in tropical regions with hot and humid climates than in temperate zones because the ecological conditions in the former enhance microbial growth and consequent biodeterioration of stone structures. Colonization on stone surfaces starts with phototrophic microorganisms forming protective biofilms enriched with organic and inorganic salts that attract heterotrophic bacteria, protozoa, fungi, and other microorganisms, which in turn form complex consortia that may penetrate into the pores or cracks of the stone up to a few millimeters. In the tropics, endolithic colonization by cyanobacteria is the major deteriogen of light-exposed monument surfaces. Most cyanobacteria that colonize these exposed surfaces possess adaptive strategies that allow them to cope with desiccation and solar radiation. The production of effective sun-blocking pigments, such as MAAs and the indol-alkaloid scytonemin, and reactive oxygen scavenging (ROS) enzymes, such as superoxide dismutase, catalase, peroxidase, and reroxiredoxin, allow cyanobacteria to colonize highly sun-exposed habitats. The ability of cyanobacteria to adhere is mediated by the production of extracellular polymeric substances under desiccation stress, and exposure to high solar insolation leads to dwindling, erosion, ion leaching, and pitting of stone structures. Moreover, in the tropics these stone-colonizing cyanobacteria trap moisture, remaining viable in a dry state on the exterior surfaces of stones for most of the year. Upon wetting on the onset of the monsoon, they regain their metabolic activity and start to induce damage to the substratum, causing deterioration of even gigantic stone monuments.

Beside the damage these organisms cause to the surface of monuments of architectural and archaeological importance is the stain they leave, which requires costly cleaning. Although the conservation of these important monuments is of high economic value, only a few attempts have been made to develop suitable chemical and/or physical methods to control these stone-dwelling organisms. Hitherto, no strategy has been developed to control the formation of epilithic biofilms on specific substrates in a range of diverse ecological niches in temperate and tropical regions. The type of microorganism that colonizes exposed stone surfaces is specific to the type of substratum and the prevailing micro-ecological conditions. Hence the response of these microorganisms to a particular algaecide varies and therefore chemicals need to be found that are specific to the local conditions and microorganisms. Cost-effective methods need to be developed for the conservation of cultural heritage.

The ecology of cyanobacterial biofilms on stone monuments is not precisely understood, although it is of great importance and could provide useful information for developing effective control methods for the conservation of the cultural heritage. Engineers and architects need to develop awareness of deteriogenic micro-organisms so that future designs use materials that are less prone to biofilm formation or that incorporate biocides. It is the prime and immediate interest of the conservation sector to address intervention and conservation methods. In order to be able to achieve this it is necessary to have precise information about cyanobacterial biodiversity in the biofilms, so that effective control methods can be developed. Biochemical and biophysical studies of biopolymers exuded by stress-tolerant cyanobacteria and stress-responsive genes and enzymes are required in order to understand stress response in these organisms. Generating information from functional genomics, the production of biopolymers and the production of secondary metabolites from extremophilic cyanobacteria are new avenues for harnessing economic benefit from these organisms.

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PART III
Cyanobacterial products

Chapter 6

Therapeutic applications of cyanobacteria with emphasis on their economics

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6.1 Introduction

Cyanobacteria, a group of Gram-negative oxygenic autotrophs, are one of the oldest and most successful life forms present on the Earth (Gademan and Portman, 2008), comprising more than 150 genera and 2000 species (Hoek *et al*., 1995). Cyanobacteria represent an untapped bioresource for a diverse range of secondary metabolites (Namikoshi and Rinehart, 1996), including biologically active compounds produced during primary and secondary metabolism (Raja *et al*., 2008). These compounds show anti-algal, antifungal, antibacterial, antiviral, antilarval, antiprotozoan, antihelmintic, antimitotic, anticancer, anticoagulation, and hemagglutinating properties (Harrigan *et al*., 1998; Sponga *et al*., 1999; Tan, Marquez and Gerwick, 2002; Tan, Sitachiitta, and Gerwick 2003; Newman and Cragg, 2004; Paul *et al*., 2005; Kumar, Lakshmanan, and Kannaiyan, 2005; Tan, 2007; Jaiswal, Prasanna, and Singh, 2008), which could be utilized commercially (Moore, 1996).

Different activities in different extracts suggests different compounds with different polarities (Mayer and Gustafson, 2003). For example, *Spirulina* (*Arthrospira*) is a good natural cancer-fighting agent. Moreover, supplementation of *Spirulina* spp. has helped in stabilizing blood sugar, reducing diabetes risk (Parikh, Mani, and Iyer *et al*., 2001), fighting Candidites (El-Sheekh *et al*., 2010), reducing inflammation (Selmi *et al*., 2011), and in preventing eye diseases (Yang *et al*., 2012). Similarly, *Aphanizomenon flos-aquae* immuno-potentiates humans by triggering the migration of nearly 40% of circulating natural killer cells (within 2 h), and has stimulated the mobilization of T and B lymphocytes

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(Jensen *et al.*, 2000). The migration of natural killer cells is more pronounced in long-term consumers than the naïve subjects. There are many good articles describing the biosynthesis and mode of action of cyanobacterial bioactive compounds (Gademan and Portman, 2008; Jones *et al.*, 2010; Tan, 2010). In this article, the therapeutic potential (not other bioactive potentials) of cyanobacteria is briefly reviewed, with major emphasis on the economics of their industrial-level production.

6.2 Bioactive compounds from cyanobacteria

Cyanotoxins are a diverse group of secondary metabolites produced by many species of cyanobacteria (Codd *et al.*, 2005). For details refer to Chapter 16. The therapeutic value of cyanotoxins has been little explored, especially their antiviral, antimicrobial, and antifungal properties. However, compounds exhibiting cytotoxic activity may prove important as anticancer compounds (Sirenko, Kirkpenko, and Kirkpenko, 1999).

Apart from cyanotoxins, cyanobacteria produce a wide variety of bioactive compounds, including 40% lipopeptides, 5.6% amino acids, 4.2% fatty acids, 4.2% macrolides, and 9% amides. The majority of the bioactive compounds isolated from cyanobacteria tend to be lipopeptides (66%) with different activities: cytotoxicity (41%), antitumor (13%), antiviral (4%), and antibiotic (12%), with the remaining 18% being antimalarials, antimycotics, multi-drug-resistance reversers, and immunosuppressive agents (Burja *et al.*, 2001). Some of the compounds function as protease inhibitors, and belong to any of the following five classes: micropeptins, aerugenosins, microginins, anabaenopeptins, or microverdins. The protease inhibitors like cyanopeptolins are specific inhibitors of serine proteases, and can be used in the treatment of lung emphysema (mediated by excessive action of elastase). Cyanobacterial exopolysaccharides (CEPSs, see Chapter 12 for detail) show antiviral and immunomodulatory properties, and can have applications in medicine (Hayashi *et al.*, 1996), as well as in drug delivery. Dixit and Suseela (2013) have provided a detailed list of different cyanobacterial molecules with their potential bioactivities.

6.2.1 Cytotoxic activity of cyanobacterial metabolites

Cryptophycins (produced by *Nostoc* sp.) could be potential drugs against cancer (Barrow *et al.*, 1995). They act as mitotic inhibitor by blocking the cell cycle at the G_2/M phase via inhibition of tubulin polymerization (Panda *et al.*, 2000). Other molecules such as curacin A and dolastatin 10 have been in preclinical and clinical trials as potential anticancer drugs. Curacin A, obtained from *Lyngbya majuscula* is an anti-proliferative (antimitotic) agent and inhibits the growth of colon-, renal-, and breast-cancer-derived cell lines (Gerwick *et al.*, 1994). It is a potent competitive inhibitor of colchicine, and binds more tightly to tubulin than colchicine (Verdier-Pinard *et al.*, 1998). Cyanobacterial lipopeptides such as symplostatin 3, lyngbyastatin 3, hectochlorin, and lyngbyabellins (114-116 and 118-123) have been reported to target eukaryotic cytoskeletal macromolecules such as actin and microtubule filaments (Spolaore, Joannis, and Isambert, 2006).

Besides targeting microtubules, many cyanobacterial compounds have different but specific cellular targets. For example, Apratoxin A (126) attenuates the fibroblast growth factor signaling pathway (Lehane and Lewis, 2000) and aeruginosin (a peptide), isolated from *Microcystis viridis* (NIES-103) inhibits thrombin. Studies have shown that the sulfated polysaccharide, calcium spirulans, inhibits tumor invasion and metastasis (Mishima *et al.*, 1998) of melanoma cells. Tolyporphin extracted from *Tolypothrix nodosa* exhibits photosensitizing activity against tumor cells, and is 5000 times more effective than photodynamic treatment (photofrin II) (Morliere *et al.*, 1998). Another promising anticancer compound is borophycine, a boron-containing polyketide isolated from *Nostoc linkia* (Hemscheidt *et al.*, 1994).

In addition, these molecules lead to the development of synthetic analogues (e.g., TZT-1027, ILX-651, and LU-103793). These synthetic analogues are usually associated with improved pharmacological and pharmacokinetic properties for the treatment of different types of cancers, and are under trial (Mita *et al.*, 2006). The cancer-preventive properties of cyanobacteria in humans has been demonstrated on tobacco-induced oral leukoplakia. Mathew *et al.* (1995) reported that

oral supplementation with *Spirulina fusiformis* resulted in complete regression of homogenous leukoplakia in 57% of subjects. After discontinuation of *Spirulina* supplementation, almost half of the responders developed recurrent lesions.

Tan (2010) has reviewed the potential importance of filamentous marine cyanobacteria in drug discovery. Hundreds of secondary metabolites of cyanobacteria have been synthesized (Gademan and Portman, 2008) and identified through gene analysis (Jones *et al.*, 2010).

6.2.2 Antimicrobial compounds from cyanobacteria

An alkaloid (+)-8-hydroxymanzamine A (a complex heterocyclic ring system attached to a β-carboline moiety) first isolated from a sponge *Puchypellium* sp. and then from the *Petrosiidae* genus, exhibites potent activity against *Mycobacterium tuberculosis*, with a minimum inhibitory dose of 0.91 g/ml (Donia and Hamann, 2003). Fischerindoles (indole alkaloids) isolated from *Fischerella musciola* show anti-mycotic properties (Gademan and Portman, 2008).

Calothrixins A and B from *Calothrix* sp. inhibit the growth of the malarial parasite, *Plasmodium falciparum* and human HeLa cancer cells (Rickards *et al.*, 1999). The violet pigment nostocine A, produced by *Nostoc spongiaeforme*, exhibits cytostatic activity against human tumor cell lines (Hirata *et al.*, 1996). Nostocyclamide isolated from *Nostoc* sp. shows antibiotic activities (Todorova and Juttner, 1995).

6.2.3 Anti-viral and anti-HIV activities of cyanobacterial compounds

Bauerines A–C are indole alkaloids isolated from *Dichtrix baueriana*, and exhibit activity against the Herpes simplex virus (HSV-2) (Larsen, Moore, and Patterson, 1994). The extracts of *Lyngbya lagerheimii* and *Phormidium tenue* were found to protect human lymphoblastoid T-cells from the cytopathic effect of HIV infection. A new class of HIV-inhibitory compounds called sulfonic acid containing glycolipids has been isolated. The pure compound is active against the HIV virus in the p24 viral protein and syncytium formation assays (Gustafson *et al.*, 1989). Cyanovirin-N irreversibly inactivates diverse strains of HIV and inhibits cell-to-cell and virus-to-cell fusion. Boyd (1997) reported a novel sulfated polysaccharide, calcium spirulan (Ca-SP), as being an antiviral agent. This compound appears to selectively inhibit the penetration of enveloped viruses (Herpes simplex, human cytomegalovirus, measles virus, mumps virus, influenza A virus, and HIV-1) into host cells, thereby preventing replication (Hayashi *et al.*, 1996; Hayashi and Hayashi, 1996). A review of anti-HIV activity of cyanobacterial extracts was published by Schaeffer and Krylov (2000). Reshef *et al.* (1997) reported a sulfoquinorosyldiacyl glycerol derived from cyanobacteria as being an effective agent against HIV. Sulfolipids, a new class of anti-HIV compound from *Lyngbya lagerheimii* and *Phormidium tenue* inhibit syncytium formation upon HIV infection (Gustafson *et al.*, 1989). *Nostoc ellipsosporum* transformed with cyanovirin coding nucleotide sequences produces virucide proteins useful in HIV treatment.

6.2.4 γ-Linolenic acid

The lipid content in cyanobacteria is generally low but *Spirulina* sp. contains 6–13% lipid on a dry weight basis, half of which is fatty acids (Cohen, 1997). Gamma-linolenic acid (GLA) is a rare polyunsaturated fatty acid with a range of medicinal properties. It is 170-fold more effective than linoleic acid, the major constituent of most polyunsaturated oils. Cyanobacteria such as *Spirulina platensis* and *Arthrospira* sp. are relatively rich in GLA, which in the human body is easily converted into arachidonic acid and thence into prostaglandin E2 (Euler and Eliassen, 1967). Prostaglandins, the master hormones, control many body functions. For instance, prostaglandin E1 is involved in regulating blood pressure, cholesterol synthesis, inflammation, and cell proliferation (Horrobin and Huang, 1983; Richmond, 1988). Prostaglandin E2 is involved in contraction of smooth muscles. GLA is effective for the treatment of atopic eczema (Biagi *et al.*, 1988), premenstrual syndrome (Horrobin, 1983), heart diseases (Kernoff *et al.*, 1977), Parkinson's disease, and multiple sclerosis (Dyerberg, 1986). It lowers low-density lipoprotein in hypercholesterolemic patients (Cohen, 1997).

Aphanizomenon flosaquae contains significant amounts of ω-3-α-linoleic acid, which inhibits the formation of inflammatory prostaglandins and arachidonic acid (Kushak *et al*., 1999). Production of GLA in cyanobacteria is regulated by temperature, light intensity, growth phase, and the nitrogen status of the medium (Cohen 1997). Studies focusing on the optimization of GLA production are much needed.

6.2.5 Pigments

Cyanobacterial pigments, especially phycobiliproteins (phycocyanin, allophycocyanin, and phycoerythrin; refer to Chapter 13) show strong anti-oxidant and anti-inflammatory properties (Subhashini *et al*., 2004; Bhaskar, Gopalswamy, and Raghu, 2005). Due to their quenching action on reactive oxygen species, all antioxidants have intrinsic anti-inflammatory properties. The significant amounts of β-carotene, lycopene, and lutein provide good antioxidant properties to the cyanobacteria. Cyanobacterial phycocyanin (CPC), which represents up to 15% of the dry weight of a cyanobacterium also exhibits neuroprotective and hepatoprotective effects, besides its anti-inflammatory activity (Reddy *et al*., 2000). CPC selectively inhibits cytochrome oxidase 2 (COX-2), which is overexpressed in breast cancer cells; it has no effect on COX-1 (Reddy *et al*., 2000). CPC can be used in the treatment of Alzheimer's and Parkinson's diseases (Rimbau *et al*., 2001), and has prevented oral and skin cancers. Moreover, it can boost the immune system and possibly lower the risk of heart disease, prevent onset of cancers, and protect against age-related diseases such as cataracts, macular degradation, and multiple sclerosis (Rissanen *et al*., 2002).

6.3 Therapeutic role of whole-cell cyanobacteria (unspecified products)

Cyanobacterial supplementation helps in curing adopohepatosis (via lipoprotein lipase, an enzyme for metabolism of triglyceride-rich lipoproteins) caused by a high-cholesterol diet (Iwata, Inayama, and Katoh, 1990). *A. flos-aquae* has also been shown to inhibit the activity of maltase and sucrase in the digestive tracts of rats (Kushak *et al*., 1999). Consumption of *A. flos-aquae* is effective in accelerating recovery from mild traumatic brain injury (Valencia and Walker, 1999). Cyanobacterial consumption may have immuno- and biomodulatory effects on humans. Oral doses of *A. flos-aquae* to healthy humans produces a slight decrease in the phagocytic activity of polymorph nucleated cells (Jensen *et al*., 2000). When taken with or before a potential antigen threat, cyanobacteria may enhance IgA levels to protect against food allergies (Hayashi *et al*., 1998). This property could be useful in the treatment of diseases involving suppressed immune systems and responses such as allergies, autoimmune diseases, and chronic inflammatory conditions. It has been reported that by injecting the cyanobacterial extract intraperitoneally an hour prior to allergic challenge, mortality induced by the anaphylactic compound decreases (Yang *et al*., 1997; Kim *et al*., 1998).

6.4 Economics of cyanobacterial therapeutic products

Many cyanobacteria-based therapeutic products have potential bio-industrial futures. However, their development and commercialization is quite costly. An approach to evaluating the commercial potential of a particular product is to model the whole process to determine the approximate production costs and to assess which factors are most crucial to the final product cost. Thus to make these promising molecules cost effective requires a combination of increasing conversion efficiencies and reducing production costs. There are three major steps involved in taking a product from its natural system to market (i.e., cyanobacteria to market), each affecting the overall cost of production.

6.4.1 Screening of new strains, defining their culture condition, and mass cultivation

Cyanobacteria represent a novel and largely unexplored source of therapeutic products. There is a need

to survey new species. One such area is the survey of cyanobacteria from extreme habitats. Because of their specific requirements, their mass culture is likely to be free from contamination by other microbes (John *et al.*, 2011). Any product could have desirable and undesirable features. Thus cyanobacterial products should be assessed and prioritized according to certain criteria.

6.4.2 Methods used for isolation and purification of novel molecules

There are various methods used for the extraction of target molecules. The extraction can either be simultaneous (extra- and intracellular metabolites) or sequential (intracellular metabolites only). In both types of extraction, the first step is quenching with liquid nitrogen to freeze the metabolic activity. In simultaneous extraction, quenching is immediately followed by extraction with a suitable solvent. In sequential extraction biomass separation is done after quenching, followed by collection of the various fractions, extracting with solvents of decreasing polarity (Dixit and Suseela, 2013). Figures 6.1 and 6.2, originally drawn by Dixit and Suseela (2013), shows the steps and techniques involved in extraction and purification of bioactive compounds from cyanobacteria.

6.4.3 Clinical trials and approval

The drug-development process moves through various phases of clinical trials. For each phase, approval from The Federal Drug Administration is required. A new formulation is first tested in laboratory animals (pre-clinical phase; phase 0), and subsequently on healthy humans. The number of subjects increases with each phase: in phase I, 20–80; phase II, up to 300; phase III, up to 3000. If the drug successfully passes through all the phases, the National Regulatory Authority approves its use by the general population. Dolastatins (now dropped from a phase II trial), cryptophycins, and curacin are some of the potential anticancer cyanobacterial molecules currently at different phases of clinical trials. The whole process is costly and takes a long time (Dixit and Suseela, 2013).

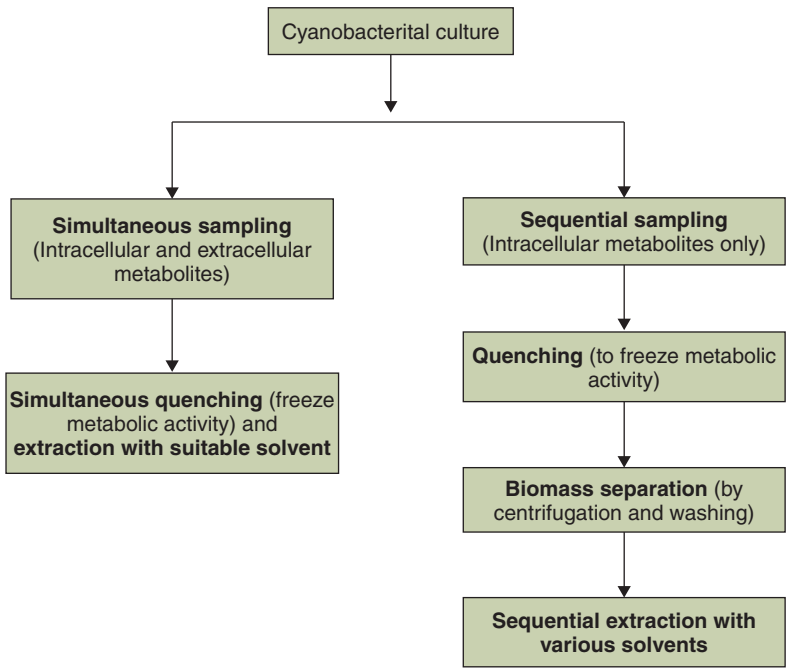
Cyanobacterital culture
Simultaneous sampling (Intracellular and extracellular metabolites)
Sequential sampling (Intracellular metabolites only)
Simultaneous quenching (freeze metabolic activity) and extraction with suitable solvent
Quenching (to freeze metabolic activity)
Biomass separation (by centrifugation and washing)
Sequential extraction with various solvents

Figure 6.1 A general extraction procedure followed for the isolation of bioactive compounds from cyanobacteria (adapted from Dixit RB and Suseela MR (2013). Reproduced with kind permission from Springer Science and Business Media).

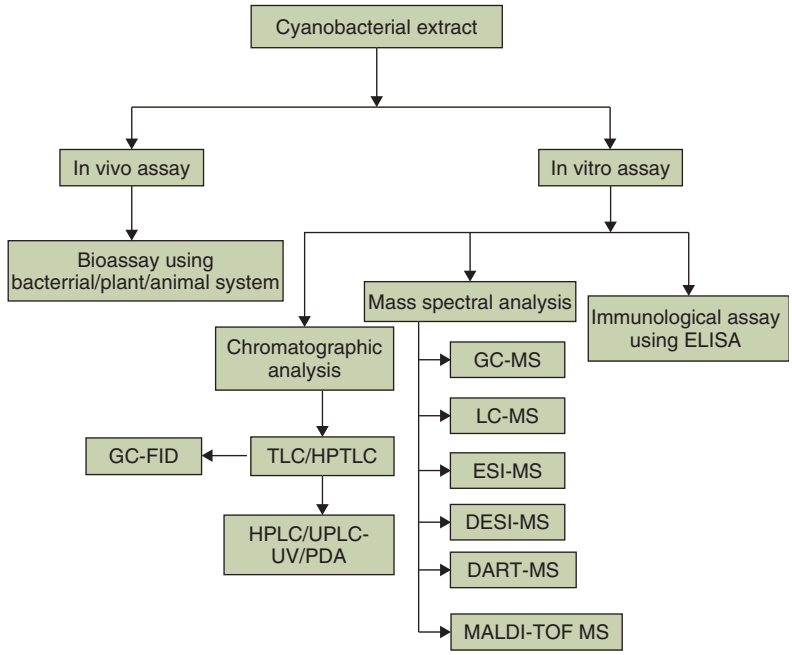

Figure 6.2 Detection and purification methods commonly employed to isolate bioactive molecules from cyanobacterial extract (adapted from Dixit RB and Suseela MR (2013). Reproduced with kind permission from Springer Science + Business Media).

Compared to other bacteria, the growth rate of cyanobacteria is relatively slow. Over-expression of the genes concerned in cyanobacterial species only slightly increases the levels of products, which suggests that more complex metabolic alterations may be required to increase yields. After evaluating the market, the cyanobacterium, the product, and the possible culture system, a more detailed strategy can be developed for commercializing the cyanobacterium and the product (Borowitzka, 1992).

Furthermore, we have to evaluate the cost of downstream processes such as the cost of processing the biomass and purified product, the costs of packaging and marketing and so on. When the product is a fine chemical these costs can be very high. The use of fertilizers may contribute to the cost of cyanobacterial biomass production. For example the use of fertilizer constitutes nearly half of the overall cost of *Spirulina* cultivation (Markou and Georgakakis, 2011). Use of wastewater rich in nitrogen and phosphorus can cut the cost of fertilizer, while at the same time cyanobacteria can reduce the inorganic and organic load of these wastewaters, thereby providing a method of biological wastewater treatment (Mallick, 2002; Mulbry *et al.*, 2008; Posten and Schaub, 2009; de-Bashan and Bashan, 2010). Provision for interest costs add another 7–10% to the product cost.

Harvesting of biomass from the broth is thought to contribute 20–30% of the total cost of biomass production (Molina Grima *et al.*, 2003). Use of filamentous cyanobacteria may help to reduce the harvesting cost, as they may be harvested easily by filtration. In addition, some filamentous cyanobacteria form aggregates and can be harvested by sedimentation or by flotation (Pouliot *et al.*, 1989; Hori *et al.*, 2002). The ability of cyanobacteria to grow in a variety of locations could be exploited to allow the production of compounds near the site of use, which could reduce transport costs.

An economic analysis of microalgal biomass production considers a number of scenarios and the effect of changing individual parameters. The major conclusions are:

- the biochemical composition of the biomass influences the economics; in particular, increased lipid

content reduces other valuable compounds present in the biomass
- the biorefinery approach (coupling the production of many products) is economically viable
- the hardest issue in assessing the economics is the cost of carbon dioxide supply.

The aim was not to provide an exact cost of the products, but to highlight the areas that are important in the economics for various management scenarios. There is a pressing need for research and development (Williams and Laurens, 2010), which is an important consideration for all bioproducts. Research and development costs of at least 9% of the final market price is probably conservative and would be much higher for small start-up company. To make cyanobacteria a commercial source of bioactive compounds, economic feasibility needs to be achieved.

6.5 Concluding remarks

The potential of cyanobacteria drug sources is well recognized and opens a new era of research. However, such products are at different stages of clinical trials, and only a few compounds have reached to the market. The cost of developing and delivering a new cyanobacterial therapeutic molecule is high, and is subject to stiff competition (where it is a variant) from synthetic molecules already present in the marketplace. The economics play a vital role in the production of these therapeutic products at industrial scales. There is a paucity (often available only in fragmented form) of information on factors key to the assessment of the economic costs involved. Therefore, to exploit these new opportunities, it is necessary to:

- screen new potential species/strains with sufficient information about their environment
- address the issues related to industrial-scale culturing
- develop new and efficient isolation and purification methodologies for the molecule of interest.

Moreover, we have to expedite the complex and lengthy but necessary process of drug testing. Thus there is a need to decrease the costs of cyanobacterial production, technological developments, and integration of different processes such as co-production of compounds with commercial value.

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Chapter 7

Spirulina: an example of cyanobacteria as nutraceuticals

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7.1 What is a nutraceutical?

Food is essential not only for growth, multiplication, and to maintain the human body but also for a healthy life. In ancient times, people that established civilizations in India, Egypt, and China were intelligent and recognized food as a kind of medicine that supports human health. Ayurveda, the earliest literature on Indian medical practice, which appeared during the Vedic period in India about 5000 years ago, mentioned the benefits of food for therapeutic purposes (Wildman, 2001). The ancient Greek Hippocrates, sometimes called the father of Western medicine, stated "Let food be thy medicine and medicine be thy food" (Bagchi, 2006).

Recent advances in modern health sciences have again highlighted the importance of food from a functional and medicinal point of view. In 1989, DeFelice, the founder of the Foundation of Innovation Medicine (FIM), New Jersey, coined the term "nutraceutical" as a combination of the words "nutrition" and "pharmaceutical" (DeFelice, 1989). He defined a nutraceutical as "a food or part of a food that provides medical or health benefits, including the prevention and/or treatment of a disease" (Bland, 1996). It has been claimed that nutraceuticals must not only supplement the diet but should also aid in the prevention and/or treatment of disease and/or disorder (Kalra, 2003).

There are many terms describing the usefulness of foods as medicinal products, for example functional foods, medicinal foods, phytonutrients, phytomedicines, and dietary supplements. These include genetically engineered foods, herbal products, and processed foods such as cereals, soups, and beverages. They are commercially available in medicinal forms, alongside a statement about a physiological benefit or protection against a chronic disease.

The boundary between phytonutrition and phytotherapy has become obscure, with the discovery that certain food components can not only modify

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physiological functions but also aid in medical practices such as drug delivery. For instance, naringenin, the 4′, 5′, 7-hydroxyflavanone found exclusively in grapefruit can slow hepatic degradation of prescription drugs such as cyclosporine, and thus potentially helps to prevent rejection of transplanted organs (Bland, 1996).

Functional foods are produced in a form close to their natural state, unlike dietary supplements, which are manufactured in liquid or capsule form. Functional foods have been either enriched or fortified, a process called nutrification. This practice restores the nutrient content in a food back to levels similar to those observed before the food was processed. The following functional foods are known to provide health benefits in addition to their basic nutritional values as food:

- green tea is known to be rich in vitamin C, and has antioxidant activity
- alfalfa is accepted as being a mineral-rich food, containing vitamins A and C, thiamine, riboflavin, niacin, calcium, iron, magnesium, and potassium.

A dietary supplement is a product that contains food-product-derived nutrients concentrated in liquid or capsule form. The US Dietary Supplement Health and Education Act (DSHEA) of 1994 defined in general terms what constitutes a dietary supplement:

> A dietary supplement is a product taken by mouth that contains a dietary ingredient intended to supplement the diet. The dietary ingredients in these products may include vitamins, minerals, herbs or other botanicals, amino acids, and substances such as enzymes, organ tissues, glandulars, and metabolites. Dietary supplements can also be extracts or concentrates, and may be found in various forms such as tablets, capsules, soft gels, gel caps, liquids, or powders.

In the United States, dietary supplements do not have to be approved by the U.S. Food and Drug Administration (FDA) before marketing. Although supplements claim to provide health benefits, products usually include a label that says: "These statements have not been evaluated by the Food and Drug Administration. This product is not intended to diagnose, treat, cure or prevent any disease." In Japan, all functional foods must meet three requirements: first, food should be present in its naturally-occurring form, rather than in a capsule, tablet, or powder; second, food should be consumed in the diet as often as daily; and third, food should regulate a biological process in the hope of preventing or controlling disease (Hardy, 2000).

The terms "nutraceutical" and "functional food" are defined by the Bureau of Nutritional Sciences of the Food Directorate of Health, Canada as follows (Therapeutic Products Programme and the Food Directorate from the Health Protection Branch, 1998). A nutraceutical is a product isolated or purified from food that is generally sold in medicinal forms not usually associated with food. A nutraceutical is demonstrated to have a physiological benefit or provide protection against chronic disease. A functional food is similar in appearance to, or may be a conventional food, is consumed as part of a usual diet, and is demonstrated to have physiological benefits and/or reduce the risk of chronic disease beyond basic nutritional functions.

Thus, the term "nutraceutical" should be considered a broad umbrella term used to describe any product derived from food sources that provides health benefits in addition to its basic nutritional value. Nutraceuticals also claim to prevent chronic diseases, improve health, delay the aging process, and increase life expectancy. In contrast to the natural herbs and spices used as folk medicine for centuries throughout Asia, the nutraceutical industry has grown alongside the expansion and exploration of modern technology (Shibamoto *et al.*, 2008).

7.2 Cyanobacteria as food

Cyanobacteria have been used as foods from ancient times. For example, *Spirulina* has been reported to be an ordinal food around Lake Chad in central Africa and Lake Texcoco in Mexico. In 1940, the French phycologist P. Dangeard described a food obtained from the market in a small village located approximately 50 km east of Lake Chad (Dangeard, 1940). He reported that the material, called Dihé in the local language, was eaten by the native population, and described how mats of microscopic algae, floating on the surface of small lakes or ponds around Lake Chad, were collected and dried under the sun on the sandy shores. On studying samples of Dihé, Dangeard concluded that it was

"a filamentous, spiral shaped blue alga." A colleague of Dangeard, A. Fremy, informed him that the organism had already been identified by F. Rich as the main constituent of the phytoplankton in a number of lakes in the Rift Valley of East Africa (Rich, 1931). Rich had also reported that the organism represented the main food source for the population of lesser flamingos (*Phoenicoptera*) inhabiting those lakes.

It has also been reported that before the 16th century, people in Mexico collected *Spirulina maxima* from Lake Texcoco and ate it after cooking. Moreover, in China, a cyanobacterium of the type of *Nostoc* (*Nostoc commune* Var. *flagelliforme*) has been used as an edible plant for about two thousand years (Gao, 1998). The *Nostoc* ("Facai" in Chinese) was sold on the market and prepared for consumption by cooking (Hisauchi, 1960). This *Nostoc* grows on stones in the basin of the upper reaches of the Yellow River. Facai has been reported as growing in desert areas, where it contributes to stabilizing the surface of the soil, and its removal is prohibited in order to protect the land from desertification. Cyanobacteria are also consumed as food in south-east Asia, where people have been aware of the nutritional value of cyanobacteria from ancient times.

Interesting bioactive compounds have been derived from terrestrial cyanobacteria. The best examples are the cryptophycins isolated from Indian *Nostoc* sp. strain GSV224 and the Scottish *Nostoc* sp. strain ATCC53789 (Trimurtulu *et al.*, 1994). Cryptophycins, originally described as antifungal compounds, were later recognized as efficient antitumor depsipeptides and include over 25 natural variants and several synthetic/semi-synthetic compounds (Trimurtulu *et al.*, 1994; Golakoti *et al.*, 1995; Subbaraju *et al.*, 1997; Chaganty *et al.*, 2004).

Some cyanobacteria are therefore edible, rich in nutritional components, and show therapeutic effects. However, certain cyanobacteria are toxic and one should be careful when using cyanobacteria as food. The WHO has stated that some species of cyanobacteria produce toxins that affect animals and humans. People may be exposed to cyanobacterial toxins by drinking or bathing in contaminated water. The most frequent and serious health effects are caused by drinking water containing the toxins or by ingestion during recreational water contact.

7.3 What is *Spirulina*?

Cyanobacteria perform oxygenic photosynthesis and are a large taxonomic group. Cyanobacteria are divided morphologically into unicellular and filamentous types. Many but not all species are capable of nitrogen fixation. Filamentous species are subdivided into those with or without heterocysts, differentiated cells that are the site of nitrogen fixation (Doolittle, 1982).

The name "*Spirulina*" is derived from the Latin word for "helix" or "spiral", and thus denotes the physical configuration of the organism. *Spirulina* naturally grows in high-salt alkaline water reservoirs in subtropical and tropical areas including America, Mexico, Asia, and Central Africa (Vonshak, 1997; Gershwin and Belay, 2007). This cyanobacterium is well known because of its use as a food or food supplement, and is considered an important dietary element in tropical areas. The Aztecs consumed *Spirulina* regularly (Van Tuerenhout, 2005). Lake Chad has become famous for its people's use of *Spirulina*, which is harvested from naturally grown populations and used as food (Belay, 2008). However, current taxonomy claims that use of the name "*Spirulina*" for strains used as food supplements is inappropriate, and there is agreement that *Arthrospira* is a distinct genus, consisting of over 30 different species, including *A. platensis*, *A. maxima*, and *A. fusiformis* (Castenholz *et al.*, 2007). These *Spirulina* (*Arthrospira*) species are edible, with high nutritional as well as potential therapeutic values (Vonshak, 1997; Khan, Bhadouria and Bisen, 2005; Gershwin and Belay, 2007; Karkos *et al.*, 2008).

Arthrospira platensis has unique physiological characteristics and its biology is the subject of a comprehensive book (Vonshak, 1997). *A. platensis* shows vigorous gliding motility of the filaments (trichomes) with a rotation along their long axis. Gliding is self-propulsion across a solid or semi-solid substrate without the aid of any visible flagellum or other structure (Hoiczyk, 2000). The mechanism of the gliding motility has not yet been elucidated.

A. platensis has become an important industrial organic material, used as a health supplement, a source of β-carotene, and a natural coloring agent. It was approved for treating symptoms of radiation sickness after the Chernobyl disaster in Russia (Loseva and

Dardynskaya, 1993). The presence of hydrogenase in its cells also makes this cyanobacterium a useful material for clean sustainable energy production (Amao and Nakamura, 2006).

7.4 Ecology of *Spirulina*

Spirulina also possesses ecologically valuable characteristics. It forms mats on the periphery of alkaline lakes (pH 9–11). It also shows salt tolerance, growing under concentrations 1.5-fold higher than sea water (Zeng and Vonshak, 1998). Accordingly, it often dominates in lakes with high carbonate/bicarbonate levels and high pH levels (Kebede, 1997). In addition, *Spirulina* thrives in warm waters (32–45°C), and survives at temperatures as high as 60°C. Certain desert-adapted *Spirulina* species can survive even when their pond habitats evaporate in the intense sun, drying to a dormant state on rocks as hot as 70°C. In this dormant condition, the blue-green color turns to a frosted white and the cyanobacterium develops a sweet flavor. There exists a speculative theory that the "manna" of the wandering Israelites, which appeared miraculously on rocks following a devastating dry spell, and described as tasting "like wafers made with honey," may have been a form of dried dormant *Spirulina*.

7.5 Nutritional values of *Spirulina*

Twenty-five years after the finding of Dangeard, J. Leonard rediscovered Dihé and confirmed that it was composed almost exclusively of dried mats of *S. platensis* collected from the waters of the alkaline lakes in the sub-desert Kanem area, northeast of Lake Chad. Leonard and his colleague Compére confirmed the report by Dangeard that Dihé was consumed by the local populations, and performed a series of chemical analyses of it, which revealed a high protein content, close to 50% of the dry weight (Léonard and Compére, 1967). Although the high content of protein in this organism was impressive, the amount was actually an underestimate since subsequent independent studies on laboratory-grown *S. platensis* have shown protein contents ranging from 62 to 68% of the dry weight (Clément, Giddey, and Menzi, 1967). It has been reported that before the 16th century, people from Central America, on the other side of the Atlantic Ocean, consumed another species of *Spirulina* – *S. maxima* –that grew abundantly in Lake Texcoco, near Mexico City (Clément, 1971, Durand-Chastel, 1980). The natives called this material "Tecuitlatle."

Spirulina is now expected to become a "food of the future" because of its amazing ability to synthesize high-quality concentrated nutrients more efficiently than any other alga. In particular, *Spirulina* contains high amounts of protein (over 60% of the dry weight), whereas beef contains only 22% protein. In addition, *Spirulina* has a photosynthetic conversion rate of 8 to 10%, compared to only 3% in crop plants such as soybeans. *Spirulina* also provides high concentrations of many other nutrients in an easily assimilable form (Table 7.1).

The trichomes of *S. platensis* are long compared to other cyanobacteria, measuring up to 0.5 mm in length, which is about 100 times the length of most other filamentous cyanobacteria. The prolific reproductive capacity of the organism and its tendency to aggregate in colonies means that *Spirulina* is easily harvested. These properties make it attractive for biotechnological applications.

7.6 Medicinal properties of *Spirulina*

Many authors have reported that use of *Spirulina* as a diet supplement has health benefits, for instance preventing or managing hypercholesterolemia, hyperglycerolemia, certain inflammatory diseases, allergies, cancer, and viral infections (Khan, Bhadouria, and Bisen, 2005; Karkos *et al.*, 2008; Kulshreshtha *et al.*, 2008). The beneficial effects of *Spirulina* on cardiovascular diseases, based on its hypolipidemic, antioxidant, and anti-inflammatory activities, are well known (Deng and Chow, 2010). *Spirulina* was also approved for treating symptoms of radiation sickness after the Chernobyl disaster in Russia (Loseva and Dardynskaya, 1993).

Table 7.1 Nutrient data for *Spirulina*.

Nutrient	Unit	Value (/100 g)
Proximates		
Energy	kcal	290
Protein	g	57.47
Total lipid (fat)	g	7.72
Carbohydrate, by difference	g	23.9
Minerals		
Calcium, Ca	mg	120
Iron, Fe	mg	28.5
Magnesium, Mg	mg	195
Phosphorus, P	mg	118
Potassium, K	mg	1363
Sodium, Na	mg	1048
Zinc, Zn	mg	2
Copper, Cu	mg	6.1
Manganese, Mn	mg	1.9
Selenium, Se	µg	7.2
Vitamins		
Vitamin C, total ascorbic acid	mg	10.1
Thiamin	mg	2.38
Riboflavin	mg	3.67
Niacin	mg	12.82
Pantothenic acid	mg	3.48
Vitamin B-6	mg	0.364
Folate, total	µg	94
Folate, food	µg	94
Folate, DFE	mcg_DFE	94
Choline, total	mg	66
Vitamin A, RAE	mcg_RAE	29
Carotene, beta	µg	342
Vitamin A, IU	IU	570
Vitamin E (alpha-tocopherol)	mg	5
Vitamin K (phylloquinone)	µg	25.5
Lipids		
Fatty acids, total saturated	g	2.65
14:0	g	0.075
16:0	g	2.496
18:0	g	0.077
Fatty acids, total monounsaturated	g	0.675
16:1 undifferentiated	g	0.328
18:1 undifferentiated	g	0.347
Fatty acids, total polyunsaturated	g	2.08
18:2 undifferentiated	g	1.254
18:3 undifferentiated	g	0.823
Amino acids		
Tryptophan	g	0.929
Threonine	g	2.97
Isoleucine	g	3.209
Leucine	g	4.947
Lysine	g	3.025
Methionine	g	1.149
Cystine	g	0.662
Phenylalanine	g	2.777
Tyrosine	g	2.584
Valine	g	3.512
Arginine	g	4.147
Histidine	g	1.085
Alanine	g	4.515
Aspartic acid	g	5.793
Glutamic acid	g	8.386
Glycine	g	3.099
Proline	g	2.382
Serine	g	2.998

Nutrient values and weights are for the edible part of the biomass. Data are based on the National Nutrient Database for Standard Reference of USDA (http://ndb.nal.usda.gov/).

7.7 Hypolipidemic effects

It has been reported that *Spirulina* has hypolipidemic effects on animals such as the mouse, rat, hamster, and rabbit. It is known that cholesterol forms several distinct particles with lipoproteins, such as high-density lipoproteins (HDL), low-density lipoproteins (LDL), and very low-density lipoproteins (VLDL). In the mouse, addition of *Spirulina* to a high fat and cholesterol diet decreases total serum cholesterol, LDL cholesterol and VLDL cholesterol and phospholipids but increases serum HDL cholesterol (Kato *et al.*, 1984). The hypolipidemic activity of *Spirulina* has been demonstrated in a diabetic mouse model (Rodriguez-Hernandez *et al.*, 2001). The data hitherto accumulated have shown that *Spirulina* possesses hypolipidemic

activity in humans. However, additional clinical studies with large sample size and high quality experimental design are required to confirm the hypolipidemic and hypoglycerolemic effects of *Spirulina* in the human population (Deng and Chow, 2010).

7.8 Antioxidant and anti-inflammatory effects

Oxidative stress and inflammation cause cardiovascular diseases, including atherosclerosis, cardiac hypertrophy, heart failure, and hypertension. *In vitro* and *in vivo* studies have revealed that *Spirulina* or its extracts possess antioxidant or anti-inflammatory activities, suggesting that *Spirulina* may contribute effectively to managing cardiovascular conditions. It has been reported that *Spirulina* inactivates free superoxide radicals generated during an oxidative burst in a dose-dependent manner (Dartsch, 2008). In an *in vitro* study, the antioxidant effect of methanolic extract of *Spirulina* on spontaneous lipid peroxidation of rat brain homogenate has been investigated (Miranda *et al.*, 1998). It was shown that *Spirulina* extract inhibits the production of thiobarbituric-acid-reactive substances such as malondialdehyde by almost 95%, indicating potent antioxidant activity. An anticancer drug, fluorouracil, has cardiac toxicity, and such cardiotoxicity derives from fluorouracil-induced impairment in the myocardial antioxidant defense system, leading to cardiac peroxidation (Simbre *et al.*, 2005). The protective effect of *Spirulina* on fluorouracil-induced lipid peroxidation was evaluated using liver homogenate from goat (Ray, Roy and Sengupta, 2007). It was shown that fluorouracil causes an increase in biomarkers of lipid peroxidation, and decreases the glutathione and nitric oxide content. On the other hand, *Spirulina* water extract significantly reduces the levels of biomarkers of lipid peroxidation, and increases the reduced glutathione content.

To evaluate the antioxidant or anti-inflammatory activity of *Spirulina in vivo* a number of animal experiments have been carried out. In one study, the antioxidant and anti-inflammatory activity of *Spirulina* was demonstrated in aged male rats (Gemma *et al.*, 2002). *Spirulina* exhibits a protective effect on cardiotoxicity induced by doxorubicin, an anthracyclin antibiotic, most likely through its antioxidant activity (Khan *et al.*, 2005). *Spirulina* extracts increase *in vitro* the basal synthesis and release of nitric oxide and the cyclooxygenase-dependent vasoconstricting agent prostanoid by the endothelium (Paredes-Carbajal *et al.*, 2001; Mascher *et al.*, 2006). Data from these studies demonstrate that *Spirulina* ingestion significantly relieves oxidative stress or inflammation and the associated pathological damage. Although these studies did not directly investigate the effects of *Spirulina* on cardiovascular conditions, the findings suggest antioxidant and anti-inflammatory activities of this cyanobacterium. However, in contrast to numerous studies using animals or tissues, only a limited number of clinical trials have been carried out to evaluate the antioxidant or anti-inflammatory activity of *Spirulina* in humans (Kim and Kim, 2005, Park *et al.*, 2008).

7.9 Mechanism of medicinal function

The hypolipidemic effect of *Spirulina* has been demonstrated in laboratory and clinical studies, however its mechanism of action is virtually unknown. The active ingredients in *Spirulina* that are responsible for the hypolipidemic activity remain to be identified. Phycocyanin, a water-soluble pigment protein present in cyanobacteria, is a possible candidate for an active ingredient. Ingestion of phycocyanin extracted from *Spirulina* results in a significant decrease in serum total cholesterol, whereas serum high-density lipoproteins cholesterol concurrently increases. Thus, it has been suggested that phycocyanin might be the active ingredient in *Spirulina* that is responsible for the hypolipidemic activity (Nagaoka *et al.*, 2005).

The antioxidant and anti-inflammatory properties of phycocyanin were discovered in 1998 (Romay *et al.*, 1998; Romay, Ledon, and Gonzalez, 1998) and have been confirmed by numerous follow-up studies (Gonzalez *et al.*, 1999; Romay *et al.*, 2001; Remirez *et al.*, 2002; Patel, Mishra, and Ghosh, 2006; Cherng *et al.*, 2007; Riss *et al.*, 2007). Phycocyanin inhibits formation of pro-inflammatory cytokines such as TNFα,

suppresses cyclooxygenase-2 expression, and decreases prostaglandin E(2) production (Romay *et al.*, 2001; Remirez *et al.*, 2002; Patel, Mishra, and Ghosh, 2006; Cherng *et al.*, 2007; Riss *et al.*, 2007). Besides phycocyanin, *Spirulina* contains a medicinally active ingredient, β-carotene, that might have potent antioxidant and anti-inflammatory activity. It has been reported that β-carotene has antioxidant and anti-inflammatory activities (Schafer *et al.*, 2002; Bai *et al.*, 2005; Katsuura *et al.*, 2009). In a study that compared β-carotene, vitamin E, and nitric oxide as membrane antioxidants it was found that β-carotene protects against singlet oxygen-mediated lipid peroxidation (Schafer *et al.*, 2002).

Hitherto accumulated knowledge on the underlying mechanisms for the medicinal effects of *Spirulina*, particularly the hypolipidemic effect, is still limited. Moreover, studies to identify the active ingredients in *Spirulina* and reveal the mechanism of its therapeutic effects will provide the knowledge required if new drugs for the prevention or treatment of hypercholesterolemia and cardiovascular diseases are to be developed.

7.10 Regulations and safety profile

The activities of nutraceuticals or phytochemicals that seem to promote human health must be carefully evaluated using the same risk–benefit model traditionally used to assess concentrated levels of any substance placed into the body. In countries such as the United States of America (http://www.cfsan.fda.gov/list.html), Canada (http://www.hc-sc.gc.ca/index_e.html), the European Union (http://www.emea.eu.int), China (http://www.sfda.gov.cn/cmsweb/webportal), and India (http://mohfw.nic.in/), there is strict regulation of food and drugs in terms of manufacturing, marketing, and usage (Zhao, 2007). Many countries are making laws and complementary regulations to address the health risks of nutraceuticals (Turner, Degnan, and Archer, 2005; Coppens, da Silva, and Pettman, 2006; Ohama, Ikeda, and Moriyama, 2006). In Europe, scientific risk assessment is performed by the European Food Safety Authority, and risk management by the European Commission. The Canadian government provides some guidelines for evidence-based clinical practice of clinical nutrition support and enteral or parenteral nutrition for ill adults (Heyland *et al.*, 2003).

Supplementing the diet with *Spirulina* has not been reported to have any embryotoxic effects. In a study on rats, it was concluded that *Spirulina* has no detectable adverse effect on reproductive performance, or embryo and fetus development and growth (Chamorro *et al.*, 1996). Hematologic and clinical chemical analyses revealed no abnormalities. In addition, no gross or microscopic changes were detected in a histological evaluation (Salazar *et al.*, 1998). Feeding *Spirulina* to rats for 1–12 weeks did not cause any changes in behavior, food and water intake, growth, and health status of these animals (Hutadilok-Towatana *et al.*, 2008).

Mercury and other heavy metals have been detected in *Spirulina* grown in open water (Johnson and Shubert, 1986), albeit at low levels. *Spirulina* growing in controlled water sources and commercial *Spirulina* products have been found to contain much lower levels of mercury and lead than the guidelines for daily intake of these elements by the WHO's Food and Agriculture Organization (FAO) (Slotton, Goldman, and Franke, 1989). Thus, supported by the long history of its use as food, and favorable safety profile in animal studies, *Spirulina* can generally be considered safe for human consumption. Additional clinical studies are required to establish systemically the safety profile of *Spirulina* in humans.

However, we should notice that certain cyanobacterial species produce cyanotoxins, and contamination of *Spirulina* products with these species may be deleterious for consumers (Chorus and Bartram, 1999). The cyanotoxin anatoxin-a, which has acute neurotoxicity, was detected in 3 of 39 cyanobacterial samples (Rellan *et al.*, 2009). According to a WHO statement, *Microcystis*, *Anabaena*, *Oscillatoria*, *Nodularia*, *Nostoc*, *and Cylindrospermopsis* contain hepatotoxins. Some strains of *Aphanizomenon* and *Oscillatoria* produce neurotoxins. The cyanobacterial species *Cylindrospermopsis raciborskii* may also produce toxic alkaloids that cause gastrointestinal symptoms or kidney disease in humans. Not all strains of these cyanobacteria produce toxins, but it is likely that there are as yet unrecognized toxins. Although *Spirulina* has been shown to be

non-toxic, quality control in the growth of *Spirulina* and its processing are indispensable to avoid contamination by other cyanobacteria (or other toxic algae) and guarantee the safety of *Spirulina* products.

7.11 Molecular studies of *Spirulina*

Arthrospira platensis has particular physiological characteristics and its biology is described in a comprehensive book (Vonshak, 1997). Gene expression in *A. platensis* during adaptation to new environmental conditions such as a high salt concentrations and the importance of the cAMP signaling cascade in its regulatory mechanism in response to changes in the external environment has been highlighted (Ohmori *et al.*, 2009). Moreover, molecular analysis of adenylate cyclase genes has shown that *A. platensis* develops a number of diverse cAMP-dependent signal cascades to adapt to different severe environmental conditions (Yashiro, Sakamoto, and Ohmori, 1996; Kasahara *et al.*, 2001; Ohmori and Ohmori, 2002). It should be noted that *A. platensis* has become an important industrial organic material as a health supplement, a source of beta-carotene, and a natural coloring agent. Furthermore, the presence of hydrogenase makes this cyanobacterium a useful organism for clean energy production (Amao and Nakamura, 2006).

Despite its various useful applications very little is known about the genomic system of *A. platensis*. Gene manipulation through genetic engineering to produce useful products needs to be considered. However, hitherto, genetic transformation of *Arthrospira* has had limited success (Kawata *et al.*, 2004) and thus commercial use of this organism has faced barriers. To overcome these barriers, restriction-modification systems based on its genome sequence may prove useful.

In 1996, for the first time, the whole genome sequence of the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 became available (Kaneko *et al.*, 1996). Since then, the genomes of more than 40 strains of cyanobacteria have been sequenced. Among them are four nitrogen-fixing filamentous species. The others are unicellular species, both nitrogen-fixing and non-nitrogen fixing. Thus, the sequencing of the complete genome of *A. platensis* was the first of a filamentous non-nitrogen-fixing species (Fujisawa *et al.*, 2010).

7.12 Structural features of the *A. platensis* genome

The *A. platensis* genome is composed of a single, circular chromosome of 6.8 Mb with an average G + C content of 44.3%; no plasmid DNA sequences have been detected. Upon GC skew analysis in order to locate the probable origin and terminator of DNA replication, no apparent shift of skew has been detected, as is the case in other cyanobacteria. The estimated genome comprises 6630 potential protein-coding genes (average size, 835 bp), as well as 49 RNA genes consisting of 2 sets of rRNA genes, 40 tRNA genes representing 20 tRNA species, tmRNA, the beta subunit of RNase P, and signal-recognition particle RNA. Of the 6630 potential protein-coding genes, 5157 (78%) were orthologous or had similarity to genes of known function or hypothetical genes (*E*-value of $<10^{-3}$) and the remaining 1473 (22%) showed no significant similarity to any registered genes. On manual curation, 2539 (38%) genes were assigned to biological functions. Genes for general metabolism in cyanobacteria were detected with no particular difference. It should be stressed that the biosynthesis genes for cyanotoxins such as non-ribosomal peptide toxins (microcystins), ribosomal depsipeptide toxins (microviridins), alkaloid toxins (anatoxins and saxitoxins), and urea-derived toxins (cylindrospermopsin) were not present in the genome, which explains with the long-established use of this organism as a food. It is known that the genus *Arthrospira* possesses gas vesicles for buoyancy, which is in contrast with the absence of gas vesicles in the morphologically similar genus *Spirulina* (Guglielmi, Rippka, and Tandeau De Marsac, 1993). Consequently, *A. platensis* has six gas vesicle genes (*gvpA-C-N-J, gvpV* and *gvpW*); GvpA is a structural protein that forms the skeleton of the gas vesicle.

7.13 Restriction modification system

Three sets of type I restriction modification (RM) systems (*hsdMSR*) are found in the *A. platensis* NIES-39 genome, which are shared with a closely related strain reported by a Chinese group (Zhao *et al.*, 2006; Fujisawa *et al.*, 2010). Among them, two RM-specific genes (*hsdS*, NIES39_A06660 and NIES39_C00340) are mosaically conserved in the two strains whereas the full-length methylase and restriction genes *hsdM* and *hsdR* are highly conserved (>98%). HsdS protein is mosaically composed of two target recognition domains and the N-terminal, central, and C-terminal regions. These mosaically conserved HsdS proteins may be generated by one or two homologous recombinations through a mechanism similar to domain swapping of *hsdS* genes in other bacteria (O'Sullivan *et al.*, 2000). This enables *A. platensis* to acquire the restriction system against a wider range of exogenous DNA elements, interfering with genetic transformation of *A. platensis*. Another *hsdS*-like reading frame, which is interrupted by an in-frame stop codon, is totally conserved between these two strains. NIES-39 harbors eight clusters of the type II RM systems, four of which are identical to those of the strain of the Chinese group (Zhao *et al.*, 2006), although three are frame shifted. The other four clusters are novel in NIES-39. Based on this information about the RM systems, it will be feasible to further improve genetic transformation in *A. platensis*.

7.14 CRISPR system

Clustered regularly interspaced short palindromic repeats (CRISPRs) are widespread in the genomes of bacteria and almost all archaea, and confer resistance to phages together with a group of CRISPR-associated (Cas) proteins (Haft *et al.*, 2005; Barrangou *et al.*, 2007; Sorek, Kunin, and Hugenholtz, 2008). The *A. platensis* genome contains three regions of CRISPRs, which are located in close proximity to Cas proteins (Fujisawa *et al.*, 2010). CRISPR1 is organized as 17 almost-identical sequences of 35 nt interspaced by non-identical sequences of 34–43 nt. CRISPR2 and CRISPR3 are organized as 29 and 23 identical sequences of 36 and 35 nt interspaced by non-identical sequences of 37–49 and 32–41 nt, respectively. Moreover, 18 Cas genes have been found. One of the three Cas1 proteins encoded by NIES39_F00150 is a fusion protein, having a C-terminal Cas1 domain and a reverse transcriptase domain similar to that of group II introns.

7.15 Mobile DNA elements

Group II introns include ribozymes and retro-elements in the genomes of organelles, bacteria, and archaea. In bacteria, group II introns primarily act as retro-elements consisting of six RNA structural domains (DI–DVI) and an intron-encoded protein, whereas in mitochondria and plastids group II introns frequently lack the intron-encoded protein and are mobile. At least 150 group II introns were identified in the *A. platensis* genome; 71 of these encode reverse transcriptase/maturase, while the other 79 do not (Fujisawa *et al.*, 2010). Additionally, 88 sequences were assigned to the group II catalytic intron (RF00029), DV and DVI. This number was much higher than that of previously reported abundant species (Nakamura *et al.*, 2002) (27 copies of group II introns in *Thermosynechococcus elongatus* and 28 copies of RF00029 in *Trichodesmium erythraeum*). These results suggest that group II introns have been self-propagating extensively in *A. platensis* cells for a long time.

Group I introns include ribozymes that catalyze their own RNA splicing to produce ligated exons (mature RNA) and the excised intron. Two group I introns have been detected in one gene, comprising three ORFs, which encode class I ribonucleotide reductases. Notably, a stop codon was found just after the insertion position in each intron, indicative of translational regulation. It is extremely rare for a group I intron to be inserted into protein-coding genes in bacteria, although one report mentions this insertion in class II RNR of the cyanobacterium *N. punctiforme* (Meng, Zhang, and Liu, 2007).

Novel phage-like sequences spanning from 12.5 to 25.5 kb have been found in at least 18 loci in the

A. platensis genome. Although there are a number of variations in deletion, insertion, and rearrangement in each sequence, they consist of phage infection-related genes and small conserved putative genes, generating a direct repeat in some cases. Such phage-like sequences contribute to at least 295 kb in the whole genome. As for transposases of the insertion sequence, 139 genes were found, which were similar to those in other cyanobacteria (Kaneko *et al.*, 1996).

7.16 Transcription factors

Cyanobacteria have multiple σ^{70}-type sigma factors of RNA polymerase and no σ^{54}-type sigma factor. In the *A. platensis* genome there are seven σ^{70}-type sigma factors (Fujisawa *et al.*, 2010). NIES39_L06110 encodes a group 1 sigma factor, which is also called the principal sigma factor and is essential for cell viability. In addition, three group 2 sigma factors (NIES39_A03770, NIES39_A08720, and NIES39_C01040) show a high degree of sequence similarity with the principal sigma factor but are non-essential for cell growth. The three group 3 sigma factors (NIES39_C05220, NIES39_D05870, and NIES39_O06410), which are divergent from group 1 and 2 sigma factors in amino acid sequence have been identified. The B-, C-, and D-type group 2 sigma factors and the F- and G-type group 3 sigma factors are conserved among non-marine cyanobacteria (Yoshimura *et al.*, 2007; Osanai, Ikeuchi and Tanaka, 2008); all of them are present in *A. platensis*.

In the *A. platensis* genome there are 66 putative genes for transcriptional regulators. Compared with the genome size, this number is small and is more similar to marine cyanobacteria (Wu *et al.*, 2007). For example, the freshwater cyanobacterium *Anabaena* sp. PCC 7120, having a similar genome size as *A. platensis*, contains more than 100 transcriptional regulators (Ohmori *et al.*, 2001). Some well-conserved transcriptional regulators among cyanobacteria have been found in *A. platensis*, including genes for NtcA (Vega-Palas *et al.*, 1990), NtcB (Aichi, Takatani, and Omata, 2001), NdhR (McGinn *et al.*, 2003), HrcA (Nakamoto, Suzuki, and Kojima, 2003), and CRP (Yoshimura *et al.*, 2000). Although *A. platensis* does not form heterocysts, a gene for HetR (NIES39_C03480) has been found (Zhang, Chen, and Zhang, 2009). *A. platensis* contains 20 response regulators that have a so-called receiver domain at the N-terminus and the helix-turn-helix type DNA-binding domain at the C-terminus. Of these regulators, 16 are classified in the OmpR family, whereas the remaining belongs to the LuxR family. Orthologous proteins of NrrA, ManR, RpaA, SphR, RpaB, and NblR have also been identified (Aiba and Mizuno, 1994, Ashby and Mullineaux, 1999, Ogawa *et al.*, 2002, Ehira and Ohmori, 2006).

7.17 Membrane transporters

The *A. platensis* genome contains 180 genes encoding putative membrane transporters. NapA-type Na^+/H^+ antiporters (Nha3 in *S. elongatus* PCC 7942) are known to be involved in salt tolerance at alkaline pH in some cyanobacteria (Wang, Postier, and Burnap, 2002; Laloknam *et al.*, 2006; Billini, Stamatakis, and Sophianopoulou, 2008). *A. platensis* possesses seven putative Na^+/H^+ antiporters, one of which (NIES39_C00590) is an orthologue of NapA and Nha3.

Accumulation of bicarbonate in the cytoplasm is essential for photosynthesis under high-pH conditions (Kaplan *et al.*, 2008). *A. platensis* possesses two sets of genes for modulation of the uptake of carbon dioxide (in the form of bicarbonate). The NDH-1 complexes (NdhF3-D3-CupA-CupS for high affinity and NdhF4-D4-CupB for low affinity) convert carbon dioxide to bicarbonate in the cytoplasm. Genes *sbtA* and *bicA* encode the sodium-dependent bicarbonate transporter in *A. platensis*. Two closely related copies of *bicA* (NIES39_B00700, NIES39_B00710) are tandem arranged in the genome. There is only one operon for the ABC-type transporter for either nitrate (NrtA-B-C-D) or bicarbonate (CmpA-B-C-D).

7.18 Photosynthesis-related genes

Almost all photosynthesis genes are detected in *A. platensis*. For photosystem II, the reaction-centre

D1 protein is encoded by at least three identical copies and one divergent copy of *psbA*. Additional variant genes include cytochrome c550-like (NIES39_J05860) and psb28-2 (NIES39_A01290). For photosystem I, all the known genes including *psaX* have been detected. Genes for cytochrome b_6/f, ATP synthase, and NDH are detected, as in other cyanobacteria. There are two copies of cytochrome c_6, but no typical electron carrier proteins in the thylakoid lumen such as plastocyanin and cytochrome c_M.

7.19 Genes for biosynthesis of carotenoid and lipid

All known cyanobacterial genes involved in carotenoid biosynthesis are detected in the *A. platensis* genome, except that for β-carotene ketolase (CrtW or CrtO). However, 3-hydroxyechinenone, produced by ketolase, is reported to bind to an orange carotenoid protein (NIES39_N00720) to regulate energy dissipation from phycobilisome to photosystem II (Kerfeld *et al.*, 2003; Wilson *et al.*, 2006). Therefore, a yet unknown type of ketolase may be present in the genome. All known cyanobacterial genes involved in biosynthesis of glycerolipids, fatty acids, lipoic acid, vitamin E, lipopolysaccharides, and polyhydroxyalkanoates are assigned. A gene for ω-3 fatty acid desaturase (*desB*) was not found, which is consistent with the biochemical analysis of *A. platensis* (Murata, Wada, and Gombos, 1992).

7.20 Reactive oxygen species protection

Genes for enzymes that protect against reactive oxygen species, except that for catalase, have been detected. However, the *A. platensis* genome harbors many genes for thioredoxin peroxidase, peroxiredoxin, and other putative peroxidases, including five genes for bacterioferritin co-migratory proteins.

7.21 Signal transduction proteins

The cosmopolitan distribution of *Spirulina* indicates its excellent capability to adapt to various environments. cAMP is an important signaling molecule in cyanobacteria (Ohmori and Okamoto, 2004). The *A. platensis* genome encodes 22 adenylate cyclases that produce cAMP from ATP (Fujisawa *et al.*, 2010). This high number is unusual among cyanobacterial genomes, which normally encode less than 10 adenylate cyclases. Even *T. erythraeum*, the cyanobacterium most closely related to *A. platensis*, contains only 13 adenylate cyclase genes. In addition to the genes *cyaC*, *cyaG*, *cyaA*, *cyaB1*, *cyaB2*, and *cyaD* homologues (Yashiro, Sakamoto, and Ohmori, 1996; Katayama and Ohmori, 1997; Kasahara *et al.*, 2001), many novel genes have been detected. Another unique characteristic of *A. platensis* is its 10 membrane-associated adenylate cyclase – most cyanobacteria have only one or two. Thus, the cAMP signal transduction response to external stimuli may be highly developed in *A. platensis*. As for the domain structure of adenylate cyclase, NIES39_A07100 bears a unique domain architecture: a Ser/Thr protein kinase domain in the N-terminus and GAF, PAS, and adenylate cyclase domains in the C-terminus. This domain architecture is similar to that of the HstK family (Phalip, Li, and Zhang, 2001), a hybrid of Ser/Thr protein kinase and histidine kinase, except that the C-terminal histidine kinase domain is replaced with the adenylate cyclase domain.

7.22 Two-component signal transduction systems

Two-component signal transduction systems canonically consist of two proteins: a histidine kinase and a response regulator (Stock, Ninfa and Stock, 1989). There are 84 putative genes for histidine kinases in the *A. platensis* genome (Fujisawa *et al.*, 2010). Among them, 33 encode hybrid histidine kinases containing not only a transmitter domain but also single or multiple receiver domain(s) or histidine phospho-transfer domain(s), which accept the phosphate group via intramolecular and/or intermolecular

phospho-transfer. There are also 65 putative genes for response regulators.

Orthologous histidine kinases of *Synechocystis*, Hik2 (slr1147), Hik7 (SphS, sll0337), Hik8 (sasA, sll0750), Hik33 (sll0698), and Hik34 (slr1285) are conserved in almost all cyanobacterial genomes. The *A. platensis* genome contains orthologues of Hik2 (NIES39_J01640), Hik8 (NIES39_A03740), Hik33 (NIES39_M02760), and Hik34 (NIES39_J00420), but no SphS, a kinase that induces proteins to acquire inorganic phosphate under Pi-deficient conditions. Although some marine cyanobacteria have been reported that lack apparent orthologues of SphS, *A. platensis* is the first example among freshwater cyanobacteria. Accordingly, it does not contain a gene for SphU, which regulates SphS (Juntarajumnong *et al.*, 2007). However, the response regulator SphR and its target genes *phoA* (alkaline phosphatase) and *pts* (high-affinity Pi transporter) are present in the *A. platensis* genome, suggesting a different regulation system in this cyanobacterium.

7.23 Future aspects

The potential and promising applications of stem cell research have been a hot topic in medicine. Stem-cell transplantation is assumed to cure many diseases (Gennero *et al.*, 2006). Some researchers have also investigated the effects of certain nutraceuticals on stem cell growth and proliferation, since this could stimulate endogenous stem cells to bring about healing and regeneration, as an alternative to stem cell transplantation. Bickford and colleagues studied the dose-related effect of blueberry, green tea, catechin, carnosine, and vitamin D3 on the proliferation of human bone marrow, and compared it to human granulocyte-macrophage colony-stimulating factor or the synergistic action of combinations of nutrients in promoting the proliferation of human hematopoietic progenitors (Bickford *et al.*, 2006). This represents another potential role of nutraceuticals in promoting health and healing capability of human body.

We conclude that *Spirulina* is a useful nutraceutical, which is commercially available. Knowledge of the genome sequence of *Spirulina* will open new avenues for the production of useful nutraceuticals. In particular, elucidation of the restriction modification system will enable the manipulation of gene transfer in order to manufacture useful substances that could increase the value of *Spirulina*.

Use of *Spirulina* in the International Space Station and on Mars is being considered. Because of its high alkali and salt tolerance, this cyanobacterium can be cultured without contamination with other bacteria. Therefore, it will be possible to maintain pure cultures of *Spirulina* in space, where complex human manipulation is restricted. Cultivation of *Spirulina*, which emits oxygen and consumes carbon dioxide will contribute to maintaining the environment of the space station. Thus while *Spirulina* supports human life on Earth, in future it may do so in space too.

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Chapter 8

Ultraviolet photoprotective compounds from cyanobacteria in biomedical applications

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8.1 Introduction

Cyanobacteria are among the few groups of microorganisms for which a full array of evolutionary adaptations to photoprotection – from behavioral, to physiological and biochemical – have been studied. Among these, the synthesis of dedicated secondary metabolites to function as sunscreens and antioxidants has high potential for application in the biomedical industry. In this article we review these photoprotective compounds in the light of their biology and compare them with current, synthetic alternatives, so as to establish their potential in a rigorous manner that includes industrial practices and standards.

8.2 Ultraviolet effects and photoprotection in cyanobacteria

8.2.1 Biological effects of ultraviolet radiation on cyanobacteria

Ultraviolet radiation (UVR) has been a major environmental factor for the biology of cyanobacteria throughout their evolutionary history (Garcia-Pichel, 1998). The fossil record supports claims for the widespread distribution of cyanobacteria during

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the early Proterozoic (~2.45–2.32 Gyr) or possibly earlier. Cyanobacterial-like microfossils are the earliest direct evidence for photosynthetic life until about the end of the Proterozoic (Knoll, 2008). According to Kasting (1987) and Pavlov and Kasting (2002), during this period levels of atmospheric oxygen were quite low, representing only about 10^{-5} PAL (present atmospheric level). Since the formation of stratospheric ozone depends on a photochemical reaction between molecular oxygen and short-wavelength UVR (< 242 nm), it is presumed that the Proterozoic atmosphere did not contain ozone at levels sufficient to attenuate short-wavelength UVR in the UVC or UVB range (~180–280 nm and 280–320 nm, respectively). Without an ozone layer, life was likely challenging, particularly for phototrophic organisms that require direct exposure to sunlight in order to harvest the energy required for survival. As the highest-energy wavelength region of the UVR range (Figure 8.1), UVC can cause the most damage to DNA and other cellular targets. The eventual rise of oxygen in the atmosphere brought about the formation of the ozone layer, and rendered UVC and the shortest wavelengths in the UVB range biologically irrelevant thereafter (Kasting, 1992), although it made the UVA portion of the spectrum a deleterious factor for the first time (Garcia-Pichel, 1998). UVB can also act directly on DNA by forming mutagenic pyrimidine dimers, crosslinks, and double-strand breaks. It can also damage proteins by targeting disulfide bonds and aromatic amino acids, especially tryptophan, resulting in the loss of ternary structure and function (Jagger, 1985). This part of the UV spectrum has remained of biological importance through time. Even today, UVB photoinhibition of photosynthesis is a significant factor depressing the productivity of the world's oceans (Smith *et al.*, 1992). The least energetic ultraviolet is within the UVA range, 320–400 nm. Most of the biological effects of UVA are not caused by direct absorption, as with UVC and UVB, but rather by oxidative processes through the generation of reactive oxygen species (Latifi, Ruiz, and Zhang, 2009; Tyrell, 1991). There is, however, some evidence that UVA does directly damage DNA through the introduction of 8-oxoguanine, which mismatches with adenine (Kozmin *et al.*, 2005), as well as through the formation of cyclobutane pyrimidine dimers, as suggested through atomic force microscopy studies (Jiang *et al.*, 2009). For organisms such as cyanobacteria that produce oxygen during their metabolic activity and are packed full of UVA-absorbing photosensitizing pigments, UVA can be a very important detrimental factor, and can account for most of the damage to the physiological machinery (Garcia-Pichel, 1998). In the cyanobacterium *Nostoc punctiforme* ATCC 29133, for example, adaptation to UVA potentially involves the differential regulation of 573 genes, or about 8.3% of all genes in the genome (Soule *et al.*, 2013).

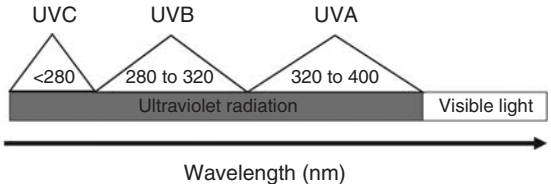

Figure 8.1 The band regions in the ultraviolet spectrum.

In addition to the direct effects of UVR on DNA and proteins, many other cellular processes are also negatively impacted in cyanobacteria (see Castenholz and Garcia-Pichel, 2012 for a review). In particular, oxygenic photosynthesis is very sensitive since the photosystem II (PSII) reaction center is a major target of UVR. PSII photochemistry provides energy from light and electrons from water oxidation, ultimately transforming light energy into chemical energy (Anderson and Styring, 1991). The D1 and D2 proteins of PSII contain the necessary cofactors for electron transport through the system (Namba and Satoh, 1987) and are targets of incident UVA and UVB radiation (Greenberg *et al.*, 1989; Frisco *et al.*, 1993). Damage to the D1 protein causes photoinhibition, a slowly reversible decrease in the rate of photosynthesis (Long and Humphries, 1994). Other major cellular processes impaired by UVR in cyanobacteria include nitrogen fixation and cell differentiation (Tyagi *et al.*, 1992; Blakefield and Harris, 1994; Castenholz and Garcia-Pichel, 2012).

8.2.2 Mechanisms of UVR damage repair and prevention in cyanobacteria

The level of exposure to UVR can vary significantly in spectral composition and overall intensity with the habitat, and is subject to variation in time and space within it (Castenholz and Garcia-Pichel, 2012).

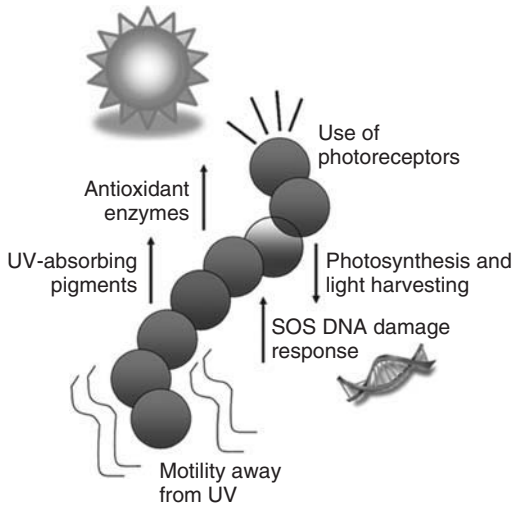

Figure 8.2 Cyanobacterial responses to UVR exposure.

In some instances, such as in soil or rock surfaces, and inter-tidal mats (Garcia-Pichel and Belnap, 1996; Rothrock and Garcia-Pichel, 2005) it can be demonstrably very high. To cope, cyanobacteria have developed an array of defense mechanisms that prevent or minimize photodamage (Figure 8.2) (Castenholz, 1997; Castenholz and Garcia-Pichel, 2012). For example, all cyanobacteria are capable of efficient DNA repair, excision repair and photoreactivation (Levine and Thiel, 1987). To cope with D1 protein damage, cells are able to replace the damaged D1 protein at a rate dependent upon the rate of damage. While the D1 protein replacement is a constant process for the cell in white light, rates of protein turnover increase in response to UVR stress (Aro, Virgin, and Anderson, 1993). Furthermore, a modified D1 PSII protein (D1:2) that is more UVB-tolerant, has been identified in a laboratory study using *Synechococcus* PCC 7492 (Porankiewicz, Schelin, and Clarke, 1998). Some cyanobacteria possess specific UVA or UVB radiation-sensing photoreceptors that are based on pterin-like chromophores (Moon *et al.*, 2010; Portwich and Garcia-Pichel, 2000). The synthesis of antioxidant proteins is also a common UV radiation shock response (Mittler and Tel-Or, 1991; Shibata, Baba, and Ochiai, 1991; Shirkey *et al.*, 2000; Soule *et al.*, 2013). Additionally, some of the motile, gliding cyanobacteria display behavioral UVR avoidance by vertical migrations (0.4–1 mm) away from the incident UV source in a variety of benthic habitats (Bebout and Garcia-Pichel, 1995; Kruschel and Castenholz, 1998; Nadeau, Howard-Williams, and Castenholz, 1999).

Aside from behavioral avoidance, all of the strategies described above are primarily repair mechanisms, deployed in response to damaged biomolecules or impaired cellular processes. These processes are energy-consuming and not preventative in nature. Preventative strategies include the ability to synthesize and accumulate photoprotective compounds that can act as either UV-absorbing sunscreens or as antioxidant scavengers of reactive oxygen generated from incident UV (Garcia-Pichel and Castenholz, 1991, 1993; Castenholz and Garcia-Pichel, 2012). These constitute the focus of this chapter.

8.2.2.1 Photoprotective compounds in cyanobacteria: UV-absorbing sunscreens

Most sunscreens function by absorbing UVR and releasing the energy captured by emission of photons of longer wavelengths or through thermal relaxation (i.e., thermal release). Some, however, may act as a physical screen that simply blocks UVR by reflection and multiple scattering, without absorption of photons. In all cases, sunscreens prevent damaging photons from reaching potential cellular targets such as the photosynthetic apparatus and genetic material (Castenholz and Garcia-Pichel, 2012). In principle, a sunscreen does not require metabolic energy to function, and therefore constitutes a passive defense mechanism. The passive nature of sunscreens provides a particular fitness benefit to those cyanobacteria in habitats of restricted growth and nutrient availability (Garcia-Pichel, Sherry and Castenholz, 1992; Brenowitz and Castenholz, 1997; Dillon and Castenholz, 2003). Well-known cyanobacteria-derived sunscreen compounds are natural products that function through absorption, and include two classes: the mycosporine-like amino acids (MAAs), most of which block UVB, and the UVA-absorbing sunscreen scytonemin (Garcia-Pichel and Castenholz, 1991; Garcia-Pichel, Wingard and Castenholz, 1993).

Scytonemin

This sunscreen is deposited as a brown pigment in the extracellular sheaths, where it accumulates to concentrations of up to 5% of the cellular dry weight

in laboratory cultures. Even with the extremely small path lengths of microbial tegumentary layers, significant sunscreen factors are thus achieved (Garcia-Pichel and Castenholz, 1991; Gao and Garcia-Pichel, 2011b). Scytonemin is lipid-soluble, has a molecular weight of 544 Da, and is an indole-alkaloid composed of a unique dimeric carbon skeleton with fused heterocyclic units formed by the condensation of tryptophan- and phenylpropanoid-derived subunits (Figure 8.3) (Proteau *et al.*, 1993; Jones *et al.*, 2011). Its conjugated double-bond distribution allows for the molecule to absorb strongly in the UVA, with a maximum around 370–372 nm *in vivo*. It can therefore effectively absorb >95% of the ambient UVA (and some UVB) radiation before it reaches the cell.

Found exclusively among the cyanobacteria, scytonemin has been reported in over 300 species, and although it is not universally present, it is represented in all of the major taxonomic groups. As a passive protective mechanism, it has been shown to protect photosynthesis from UVA damage when metabolic activity is limited (Garcia-Pichel, Sherry, and Castenholz, 1992). It is therefore common in cyanobacteria that often find themselves in a desiccated state or growth-debilitating environments (Garcia-Pichel and Belnap, 1996; Nadeau, Howard-Williams, and Castenholz, 1999). UVA induces the synthesis of scytonemin (blue light and UVB less efficiently), while red or green light will not elicit the biosynthetic response (Garcia-Pichel and Castenholz, 1991; Ehling-Schulz, Bilger, and Scherer, 1997). In some strains other stress factors, particularly desiccation, osmotic stress, and poor nutritional status, have played a role in modulating its biosynthetic induction (Dillon *et al.*, 2002; Fleming and Castenholz, 2007; Rath, Mandal, and Adhikary, 2012).

The genomic region associated with scytonemin biosynthesis was identified through the analysis of a scytonemin-deficient mutant of *Nostoc punctiforme* ATCC 29133 generated through random transposon mutagenesis. The mutation pointed to a large genomic region of 18 contiguous genes (NpR1276–NpR1259), which were split into two major groups based on their sequence similarity to known homologues and location within the gene cluster (Figure 8.4). In the downstream region of this gene cluster are several redundant copies of genes that likely encode proteins of the shikimic acid (AroG and AroB) as well as the aromatic amino acid (TyrA and TrpECABD) biosynthetic pathways. In the upstream region of this cluster were several genes whose protein functions were unknown; many of which were later annotated as *scyABCDEF* (Soule *et al.*, 2007). It has been confirmed that the redundant copies of the shikimic acid and aromatic amino acid biosynthetic genes, found within the scytonemin locus, are dedicated to supplying the initial metabolites for scytonemin

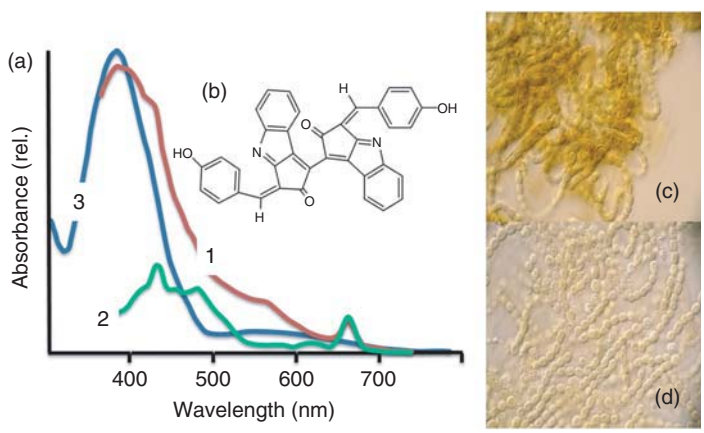

Figure 8.3 (a) Absorption spectrum of lipid-soluble pigments from the wild type *Nostoc punctiforme* ATCC 29133 (spectrum 1) and the scytonemin-deficient mutant (spectrum 2) exposed to UVA; note the absorption of scytonemin at 384 nm in the wild type. (b) The structure of scytonemin and its spectrum in solution (spectrum 3). (c) Images of the wild type and (d) scytonemin-deficient mutant exposed to UVA; note the accumulation of scytonemin as a brown pigment in C. See plate section for color version.

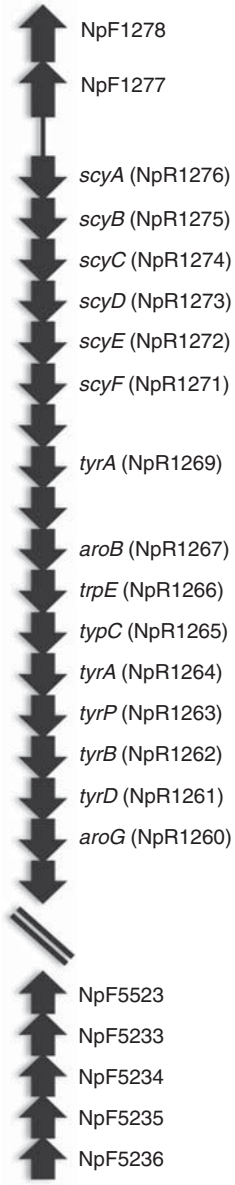

Figure 8.4 Scytonemin biosynthetic gene clusters in *Nostoc punctiforme* ATCC 29133. Both the main gene cluster and the 5-gene satellite gene cluster are shown with their orientation and loci (not drawn to scale).

biosynthesis (Soule, Garcia-Pichel, and Stout, 2009). Further characterization of this gene cluster, both within *N. punctiforme* and through comparative genomics with other cyanobacterial strains, revealed a relatively well-conserved putative two-component regulatory system (*N. punctiforme*; NpF1278 and NpF1277) and a conserved satellite cluster of five genes (*N. punctiforme*; NpF5232–NpF5236) that may also be functionally associated with scytonemin biosynthesis and metabolism (Soule *et al.*, 2009). Studies on the genetic regulation of this region confirmed the upregulation of both the main scytonemin gene cluster and the satellite cluster by exposure to UVA radiation in *N. punctiforme*, and established that the main 18-gene cluster produces a single co-transcribed unit 24.4 kb in length (Sorrels, Proteau, and Gerwick, 2009; Soule, Garcia-Pichel, and Stout, 2009). Balskus and Walsh (2008, 2009) provided crucial insights into the biosynthetic pathway of scytonemin by showing that the scytonemin precursors indole-3-pyruvic acid (I3P) and *p*-hydroxyphenylpyruvic acid (HPP) form a ketone intermediate that was previously uncharacterized. From their work it is now known that the precursors I3P and HPP are formed through the deamination of tryptophan by ScyB (NpR1275) and the oxidation of prephenate by NpR1269, respectively. I3P and HPP are then combined through an acyloin condensation mediated by ScyA to form a ketone intermediate unique to scytonemin. Further decarboxylation of this intermediate by ScyC provides what is most likely the last precursor that is synthesized in the cytoplasm. The enzymes encoded by the genes, *scyD*, *scyE*, and *scyF*, are likely involved in the later stages of scytonemin biosynthesis and may function in the periplasm as suggested by their export signal domains (Soule *et al.*, 2009).

Mycosporine-like amino acids

The second major class of sunscreens found among the cyanobacteria is the mycosporines, also referred to as the mycosporine-like amino acids (MAAs). MAAs have low molecular weight, and are colorless, water-soluble compounds composed of a core 5-hydroxy-5-hydroxymethyl-cyclohex-1, 2-ene ring with either a ketone or imino-group as the C1 substituent (Figure 8.5) (Portwich and Garcia-Pichel, 2003). All MAAs absorb within the UVB/A range through electronic transitions in their conjugated core and release the excess energy as heat (Conde, Churio, and Previtali, 2004). Over 20 MAAs have been formally described (Shick and Dunlap, 2002), their diversity accounted for by the C1 substitution as described above, as well as

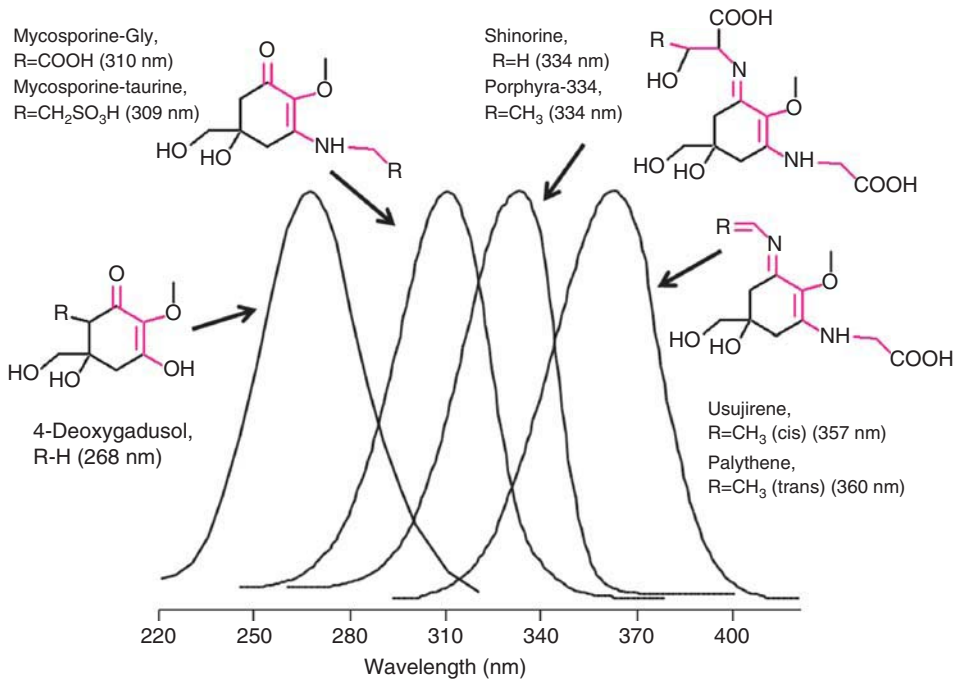

Figure 8.5 The mycosporines (MAAs); absorption spectrum and structure of representative compounds. See plate section for color version.

through various amino compound moieties at C3. The C1 and C3 substituent combination provides the individual MAAs with a unique absorption maximum between 309 and 362 nm; no other absorption bands are found down to 210 nm (Garcia-Pichel and Castenholz, 1993). In cyanobacteria MAAs are sometimes constitutively expressed but most commonly induced by exposure to UVB. In at least one strain (of *Chlorogloeopsis*), osmotic stress has been shown to play a role in biosynthetic induction (Garcia-Pichel and Castenholz, 1993; Portwich and Garcia-Pichel, 1999). In this model strain, induction of MAA biosynthesis by UVB is mediated by a pterin-like photoreceptor (Portwich and Garcia-Pichel, 2000). Most MAAs accumulate as solutes in the cytoplasm, although in *Nostoc commune* they are found covalently bound to oligosaccharides in the extracellular sheath (Böhm *et al.*, 1995). Among bacteria, MAA biosynthesis appears to be restricted to the cyanobacteria. Furthermore, they can be found in some fungi, eukaryotic micro- and macro-algae, as well as in many marine invertebrates, where they are obtained through their algal-based diet and accumulate in tissues susceptible to UV exposure, such as those found in the eye (Karentz *et al.*, 1991; Sommaruga and Garcia-Pichel, 1999). In addition to being a sunscreen, it has been suggested that they may serve as compatible solutes against salt stress, protect against desiccation, or even function as a nitrogen reservoir (Oren and Gunde-Cimerman, 2007), but evidence for those other potential roles is superficial at best.

Early studies of MAA biosynthesis using metabolic inhibitors and radiotracers in fungi and *Chlorogloeopsis* PCC 6912 suggested that the mycosporine cyclohexenone core was derived from the shikimate pathway, the precursor pathway to aromatic amino acid biosynthesis. Also corroborating this conclusion was the feedback inhibition of MAA biosynthesis by tyrosine (Favre-Bonvin *et al.*, 1987; Portwich and Garcia-Pichel, 2003). Genomic mining for putative biosynthetic genes (Figure 8.6) followed by comparative genomic studies, suggested that a likely 3-dehydroquinate synthase (DHQ synthase) flanked by a putative O-methyltransferase (O-MT)

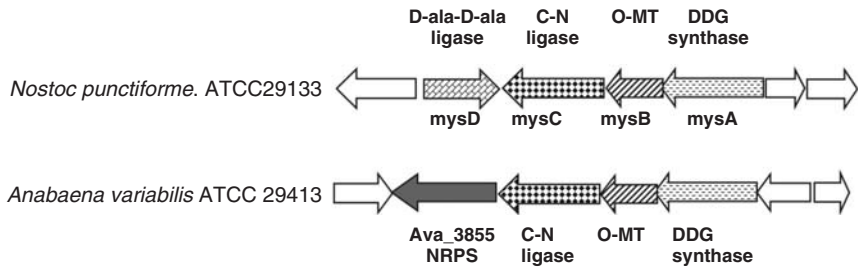

Figure 8.6 Mycosporine biosynthetic gene clusters in *Anabaena variabilis* ATCC 29413 and *Nostoc punctiforme* ATCC 29133 (not drawn to scale; based on data from Gao and Garcia-Pichel (2011a)).

in *Anabaena variabilis* ATCC 29413 (Ava_3858 and Ava_3857, respectively) could potentially constitute the biosynthetic basis to produce the cyclohexenone core of all MAAs (4-deoxygadusol; 4-DG) (Singh *et al.*, 2010). The Ava_3858 and Ava_3857 gene products (as well as the *Nostoc punctiforme* homologs NpR5600 and NpR5599) were shown to form 4-DG upon heterologous expression in *E. coli*, but only when it was supplied with sedoheptulose-7-phosphate (SHP) as a substrate, rather than the expected 3-DHQ (Balskus and Walsh, 2010). This would suggest that MAA biosynthesis does not in fact branch from the shikimate pathway proper, but rather the pentose phosphate pathway via SHP. Interestingly, when the Ava_3858 gene, also identified as sedoheptulose-7-phosphate cyclase for its homology to *2-epi-5-epi*-valiolone synthase, is knocked out in *Anabaena variabilis*, shinorine biosynthesis persists (Spence *et al.*, 2012). Thus there appears to be an alternative, yet distinct pathway for MAA biosynthesis in which the shikimate pathway provides 4-DG. Deeper into the pathway, Balskus and Walsh also confirmed that the ligation of the amino moiety (specifically glycine) occurred in an ATP-dependent manner by the gene product of Ava_3856, which is adjacent to O-MT in *A. variabilis*. The reaction was demonstrated *in vitro* when purified Ava-3856 product supplied with glycine and ATP yielded mycosporine-glycine. Furthermore, the MAA shinorine was formed when serine was provided to the purified gene product of Ava_3855, a putative non-ribosomal peptide synthase adjacent to Ava_3856 (Balskus and Walsh, 2010), thus identifying a putative 4-gene cluster in *A. variabilis* that converts SHP to the specific MAAs, mycosporine-glycine and shinorine.

8.2.2.2 Photoprotective compounds in cyanobacteria: antioxidants

While sunscreens protect cells from ambient UVR, antioxidants serve to rescue the cells once UVR has been able to elicit the formation of oxidative photoproducts. Endogenous chromophores, including the chlorophyll discussed above, as well as melanins, porphyrins, flavins, quinones, and pyrimidine nicotinamide cofactors (NADH and NADPH), will absorb UV photons, particularly UVA. The absorption of high-energy photons by these chromophores will subsequently generate reactive oxygen species (ROS), which are short-lived and extremely reactive through the non-specific oxidation of surrounding molecules. Common free radicals and ROS include molecular oxygen (O_2), superoxide anion (O_2^-), hydroxyl radical ($\cdot OH$), singlet oxygen ($^1O_2$), and hydrogen peroxide (H_2O_2) (Chaundhuri, 2005). Removal of ROS in cyanobacteria can be done enzymatically, through the use of enzymes such as superoxide dismutase (SOD) and catalase, or non-enzymatically through cyanobacteria-derived carotenoids and mycosporines (Canfield, Forage, and Valenzuela, 1992). Mycosporine-glycine in particular, is capable of removing some of these harmful ROS and can effectively reduce oxidative stress (Dunlap and Yamamoto, 1995; Suh, Lee, and Jung, 2003). Furthermore, the antioxidant α-tocopherol (vitamin E) is also produced by some cyanobacteria: it can accumulate to levels of up to 10–19 mg per 100 mg cells in *Spirulina platensis* (Babadzhanov *et al.*, 2004).

Carotenoids

The carotenoid pigments occur in many microorganisms, as well as plants and algae, in over 600 known

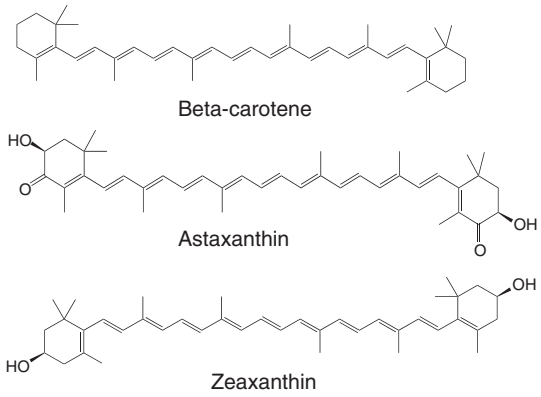

Figure 8.7 Some representative carotenoids of cyanobacteria.

varieties. They are broadly defined as tetraterpenoids with hydrocarbon chains (Figure 8.7), the latter making them lipophilic and the former enabling them to absorb visible light energy (Britton, 1995). The synthesis of some carotenoids in cyanobacteria (echinenone and myxoxanthophyll) can be induced in response to UVB radiation (Ehling-Schulz, Bilger, and Scherer, 1997). Because their absorption within the UV-range (<400 nm) is not strong, they are not especially beneficial as sunscreens. Nonetheless, they do provide some relief during high solar irradiance. This is often evidenced by the very high carotenoids-to-chlorophyll ratio, which imparts a yellow-to-orange color to cyanobacteria in regions of high light intensity (Norris, McDermott, and Castenholz, 2002). Their most valuable contribution to UVR stress, however, is based on their ability to quench reactive oxygen ($^1O_2$) and minimize triplet chlorophyll (^{3}Chl) formation through the thermal dissipation of excess excitation energy (Niyogi, 1999). In particular, β-carotene fulfills both of these purposes in cyanobacteria by absorbing energy from the excited triplet state of chlorophyll, protecting the cells against photo-oxidation (effectively competing with oxygen for the excitation energy), while also operating as an accessory pigment in the antenna complexes by absorbing light at 495 nm and 465 nm for use in photosynthesis (Hirschberg, 1994).

Mycosporine-like amino acids

In addition to functioning as a sunscreen, some of the MAAs described above seem to also serve as important antioxidant compounds. In particular, mycosporine-glycine is reactive to peroxyl radicals, which can alter membrane integrity through lipid peroxidation if left unchecked (Shick *et al.*, 2002). By scavenging these radicals, mycosporine-glycine has been shown to inhibit the oxidation of phosphatidylcholine in a concentration-dependent manner (Dunlap and Yamamoto, 1995). *In vitro* experiments have also demonstrated that the generation of $^1O_2$ by addition of the sensitizing agents methylene blue and eosin Y, decreases in the presence of mycosporine-glycine (Suh, Lee, and Jung, 2003). It is also worth noting that reduced scytonemin has also shown antioxidant properties, although this is based on one study and little has been done to follow up on these claims (Ninomiya *et al.*, 2011). Furthermore, scytonemin in its natural state, under oxic conditions, is not found in the reduced state.

8.3 Commercial sunscreens

8.3.1 Skin cancer

One of the potential biomedical applications of microbial sunscreens is in the prevention of skin cancer. The various forms of skin cancer – basal cell carcinoma (BCC), squamous cell carcinoma (SCC), and melanoma – are a result of UVR-mediated mutations in epithelial cells that become proliferative (Krutman, Ahrena, and Roza, 1996). The incidence of skin cancer cases is rising in most populations that have been followed, and according to the Skin Cancer Foundation (www.skincancer.org) over 2 million people are diagnosed annually in the United States and 1 in 5 Americans will develop skin cancer in the course of their lifetimes. Furthermore, the World Health Organization (INTERSUN Programme Fact Sheets 287 and 305, 2010) estimated that over 2 million cases of BCC and SCC and 200,000 malignant melanomas occurred globally, with over 65,000 melanoma-related deaths in 2010.

For any individual, the incidence of skin cancer is a direct function of the amount of exposure to UVR or, more precisely, of the cumulative dosage received. This depends on environmental factors such as geography and climate, on behavioral factors such as time

spent outdoors and clothing habits, and on inherent genetic factors such as skin type (Hall *et al.*, 1997; Thieden *et al.*, 2004; Hatch and Osterwalder, 2006). Of the solar energy in the UV spectrum that penetrates the ozone layer, 95% is in the long-wavelength UVA range. In addition to generating ROS, which lead to photocarcinogenesis, UVA damage to human skin cells is associated with pigmentation (tanning), photoaging, and immunosuppression (Wang, Balagula, and Osterwalder, 2010). UVA radiation can penetrate deeper than other forms of UV into the skin (Figure 8.8; ~100 μm of epidermis), where it can target melanocytes, cells that produce the skin pigment melanin (Zanetti *et al.*, 2001; Agar *et al.*, 2004). While melanin serves primarily as both a physical barrier to UVR and an absorbent filter for UVR penetration, it can also act as a photosensitizer to UVA (Brenner and Hearing, 2008). Damage to cells by UVB radiation occurs primarily through the formation of DNA lesions such as cyclobutane pyrimidine dimers (Griffiths *et al.*, 1998). These UVB-mediated mutations are generally localized to the suprabasal skin layers and serve as a causative agent for SCC, which is most often found in those individuals with a long-lasting chronic exposure to UVB (Urbach, 1997; Agar *et al.*, 2004). While UVB and UVA are both implicated in melanomas, UVB is most closely associated with non-melanoma skin cancers and in the development of erythemas, or reddening of the skin (Parrish, Jaenicke, and Anderson, 1982; Young *et al.*, 1998).

8.3.2 Sunscreens: the basics

Skin cancer prevention relies heavily on both awareness and education about the physical prevention of UVR damage by clothing and through the topical application of appropriate UVR-absorbing or reflecting chemicals such as sunscreens or sun-blocking agents. Sunscreens function by attenuating UVR through absorption, scattering, or reflection (backward scattering). In scattering, the energy transitions of the radiation and molecule are not of the same resonance frequency and the resulting dipole oscillations emit the incident radiation in multiple directions. Molecules will absorb incoming radiation when there is resonance between their energy transition and that of the radiation, resulting in the transfer of energy to the molecule. Absorption of the incident radiation excites the absorbing molecule from a ground (S_0) to an excited (S_1) electronic state. Once excited, the excess energy can be lost through fluorescence, initiate photoreactions, or be redistributed through the molecule by radiation-less transitions (Osterwalder and Herzog, 2009).

In order to qualify as a sunscreen several criteria must be met (Garcia-Pichel, Sherry, and Castenholz, 1992). At the very minimum, the molecule must absorb

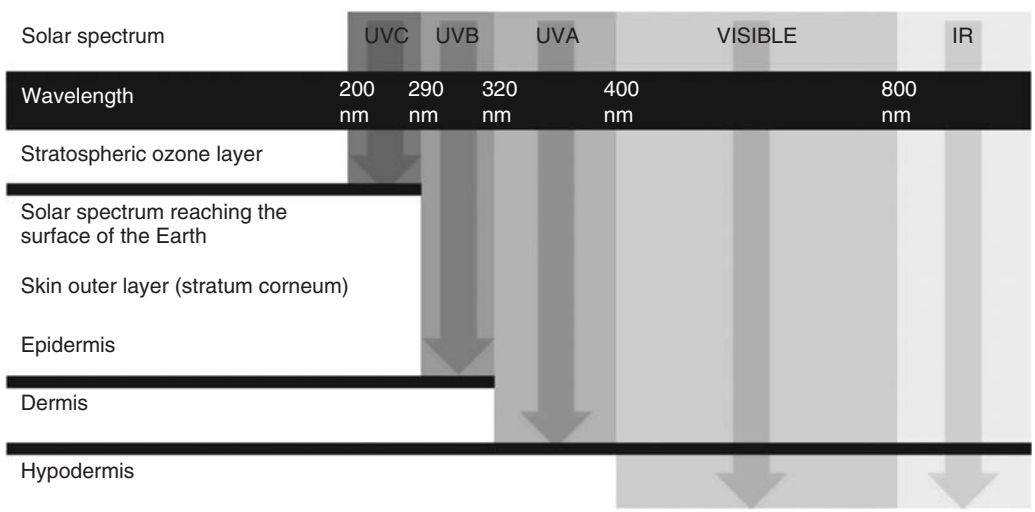

Figure 8.8 Solar radiation extinction in the environment and the human skin layers.

in the UVR range with a high absorption coefficient and its concentration must result in a significant reduction in the dose of UVR received by the target or organism. Furthermore, the use of any such sunscreen in the prevention of skin cancer should also meet several other criteria that can be more broadly considered under the headings of either efficacy or safety. Efficacy involves considerations such as the spectrum of activity, solubility, ease of incorporation into and compatibility with different formulations, photostability when exposed to UVR, aesthetics, odors, and economic practicality (Table 8.1; Osterwalder and Herzog, 2009). Safety, on the other hand, is not limited to human health but also to the environment. Sunscreen developers must consider the product's toxicity, level of irritation, photosensitization, potential effects on embryonic development, percutaneous absorption, carcinogenicity, pharmacokinetics and metabolism, and environmental contamination potential (Nohynek and Schaefer, 2001; Giokas, Salvador, and Chisvert, 2007).

8.3.2.1 Commercial sunscreens and formulations

History

Although the first commercially available sunscreen in the United States, a mixture of benzyl salicylate and benzyl cinnamate, was available as early as 1928, the first to be used widely, "Red Vet Pet" (from "red veterinary petrolatum"), was produced in 1944 by Benjamin Greene, an airman and the future founder of Coppertone®. This physical blocker was used by World War II troops with much success, but, because it was red and sticky, requiring a thick application, it was not a likely aesthetic choice (MacEachern and Jillson, 1964). In 1947, the absorption spectra for metal oxides such as zinc oxide and titanium dioxide were published, showing their strong absorption in the UVA-B range (Grady, 1947). They soon became the first inorganic UVR filters, and are still widely used today. The popular *para*-aminobenzoic acid (PABA) and salicylate derivatives such as padimate-O and octisalate became available in 1948 (Figure 8.9; Moloney, Collins, and Murphy, 2002; Roelandts, 2009, 1998). Some of the most common UV sunscreens used today still contain cinnamates, such as octinoxate and cinoxate, with octinoxate serving as one of the most potent filters in the UVB range (Palm and O'Donoghue, 2007). It was not until the 1970s, however, that the first organic UVA filters became available. They contained dibenzoylmethane derivatives, the most common of which is avobenzone (Parsol 1789®), approved for use by the European Union in 1978, and a decade later by the Food and Drug Administration (FDA) in the United States. Other UVA filters approved by the FDA include benzophenone derivatives such as oxybenzone and camphors such as ecamsule (Mexoryl SX®) (Wang, Balagula, and Osterwalder, 2010; Sambandan and Ratner, 2011).

Many sunscreens, such as ecamsule and drometriazole trisiloxane, are considered "broad spectrum" and absorb across both the UVA and UVB wavelengths

Table 8.1 Considerations in sunscreen development.

Sunscreen efficacy	Safety considerations	Other preferred qualities
Broad spectrum coverage	Acute oral and dermal toxicity	Subjective qualities
Photostable	Dermal, ocular irritation, skin sensitization	Elegant cosmetic "feel"
High substantivity	Subchronic oral and topical toxicity	Pleasant fragrance
	Chronic toxicity	
	Fertility, early embryonic development	Physical properties
	Embryofetal toxicity and peri-/postnatal toxicity	Easy, uniform application
	In vitro and *in vivo* percutaneous absorption	Nonirritating
	Topical and oral pharmacokinetic metabolism	Nonstinging
	In vitro and *in vivo* genetic toxicity	
	Carcinogenicity	Economic quality
	Photocarcinogenicity	Affordable
	Safety and efficacy in man	

Adapted from Osterwalder and Herzog (2009), Palm and O'Donoghue (2007).

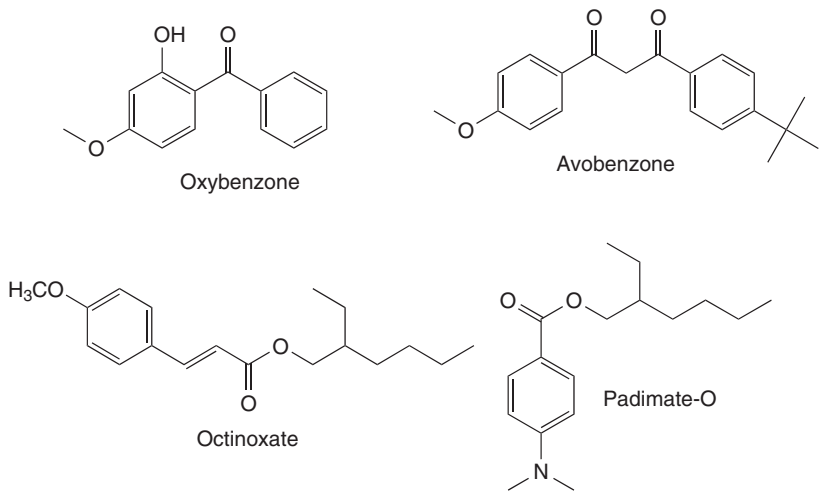

Figure 8.9 Molecular formula of some commercially-available sunscreen compounds.

(Osterwalder and Herzog, 2009; Kalia and Haiducu, 2012). The inorganic metal oxides zinc oxide (ZnO) and titanium dioxide (TiO_2) discussed above, are also considered broad spectrum. They block ambient UVA and UVB with high photostability and negligible irritation, making them among the most efficient UVA sunscreens (Agren, 1990; Tan *et al.*, 1996). There is a trend towards formulating broad spectrum sunscreens using filters effective in different regions of the UV spectra. For instance, Neutrogena's® Helioplex™ sunblock contains 3% avobenzone for UVA filtering, 10.15% homosalate and 5% octisalate for UVB filtering, and broad spectrum filters of 6% oxybenzone and 2.8% octocrylene (Table 8.2; Palm and O'Donoghue, 2007).

Sunscreen efficacy

The efficacy of a sunscreen depends on many factors, which relate to its spectrum of activity, formulation, aesthetics, and stability. One metric to gauge efficacy is the sun protection factor (SPF), which represents the fold factor of exposure length needed to obtain an equal level of redness (erythema) in protected versus unprotected skin. However, since UVB is 1000 times more erythemogenic than UVA, the SPF standard fails to define the amount of protection against UVA (Palm and O'Donoghue, 2007; Diffey and Ferguson, 2009). Several other metrics have been proposed to assess the protection conferred by a UVA sunscreen. For instance, *in vivo* methods measure the ability of a sunscreen to prevent tanning, while *in vitro* methods measure the absorbance ratio of UVAI:UV, where UVAI is defined as wavelengths from 320–340 nm (Sambandan and Ratner, 2011).

Formulation can also be a challenge to the development of an effective sunscreen. Most sunscreen formulations contain a lipid and aqueous phase. The former is usually accomplished by the addition of fatty acids, fatty alcohols, fatty esters, oils, or waxes, while the latter is achieved through the use of moisturizers, thickeners, polymers, and salts (Osterwalder and Herzog, 2009). Water-resistant properties are also desirable. This is most easily achieved through use of oil-soluble UV filters, to prevent the loss of protection during water activities or through perspiration (Diffey and Ferguson, 2009). In addition to the above additives, the formulation must also contain stabilizers or preservative agents, antioxidants, and perfumes (Osterwalder and Herzog, 2009).

An important component to sunscreen efficacy is photostability. The case of the UVA filter avobenzone is well-documented, as it will photodegrade in sunlight on its own. Because of this, avobenzone must be combined with photostabilizers in order to maximize its full absorption potential (Sayre *et al.*, 2005). Even octinoxate alone has poor photostability. Palliative measures have included its encapsulation into

Table 8.2 Sunscreen ingredients approved by the Food and Drug Administration.[1]

FDA-approved active sunscreen ingredient[2]	Peak absorption λ (nm)	Range of protection λ (nm)	Maximum FDA-approved concentration (%)
Organic filters			
UVA filters			
Benzophenones			
• Oxybenzone (benzophenone-3)	290, 325	270–350	6
• Sulisobenzone (benzophenone-4)	366	250–380	10
• Dioxybenzone (benzophenone-8)	352	206–380	3
Dibenzoylmethanes			
• Avobenzone (butyl methoxydibenzoylmethane, Parsol 1789)	360	310–400	3
Anthralates			
• Meradimate (methyl anthranilate)	336	200–380	5
Camphors			
• Ecamsule (tetraphthalyidene dicamphor sulfonic acid, Mexoryl SX)	345	295–390	10
UVB filters			
Aminobenzoates (PABA derivatives)			
• PABA (p-aminobenzoic acid)	283	260–313	15
• Padimate-O (octyl dimethyl PABA)	311	290–315	8
Cinnamates			
• Cinoxate (2-ethoxyethyl p-methoxycinnamate)	290	270–328	3
• Octinoxate (octyl methoxycinnamate)	311	280–310	7.5
Salicylates			
• Octisalate (octyl salicylate)	307	260–310	5
• Homosalate (homomenthyl salicylate)	306	290–315	15
• Trolamine salicylate (triethanolamine salicylate)	260–355	269–320	12
Others			
• Octocrylene	303	287–323	10
• Ensulizole (phenylbenzimidazole sulfonic acid)	310	290–340	4
Inorganic filters			
Titanium dioxide	Varies	290–350	25
Zinc oxide	Varies	290–400	25
Microbial sunscreens[3]			
Scytonemin	370–372	325–425	n/a
Mycosporines	Varies	309–362	n/a

[1] Adapted from Osterwalder and Herzog, 2009, Palm and O'Donoghue, 2007; [2] FDA-approved as of December, 2009; [3] These are not FDA-approved.

nanoparticles (Perugini *et al.*, 2002) and the addition of glyceridic esters (de Freitas *et al.*, 2005). Inorganic sunscreening agents such as TiO_2 can be supplemented with manganese, which acts as a scavenger of ROS and has been shown to photostabilize avobenzone and octinoxate in mixed formulations (Wakefield and Stott, 2006).

Sunscreen safety

While current formulations may provide relatively effective coverage and protection from harmful UVR, they do face some challenges. The concerns are typically related to toxicity, systemic absorption, and allergenic potential, but also, in a few cases, to carcinogenicity and other specific secondary effects such as the inhibition of vitamin D biosynthesis (Burnett and Wang, 2011). One of the most photoallergenic sunscreens is the UVB filter PABA, which has attracted the attention of the research community despite its infrequent use. PABA also appears to be carcinogenic *in vitro* through the photosensitization of thymidine dimers (Henderson *et al.*, 1994; Gasparro, Mitchnick and Nash, 1998). For this reason, it is commonly substituted with the safer, yet less effective padimate-O, itself not free of controversy (Knowland *et al.*, 1993). Other compounds capable of eliciting photoallergies include the benzophenones, of which oxybenzone has some of the highest systemic absorption levels and also affects levels of endogenous reproductive hormones (Janjua *et al.*, 2004; Antoniou *et al.*, 2008). Among the UVA filters, Parsol 1789 (avobenzone) can also cause some photoallergy, although less frequently than other sunscreens (Simeoni, Scalia, and Benson, 2004).

The inorganic reflective filters ZnO and TiO_2 present an interesting case. They cause negligible irritation, making them among the most efficient and safest sunscreens (Agren, 1990; Tan *et al.*, 1996). Unfortunately, because they whiten the skin, they are rather unattractive to consumers. In order to improve their aesthetic potential, manufacturers have produced microfine nanoparticles of these metal oxides in the size range of <100 nm. When used in low concentrations these nanoparticles are transparent on the skin. However, these particles lose effectiveness, and can potentially be absorbed by the host tissues. (Antoniou *et al.*, 2008; Nash, 2009; Pan *et al.*, 2009). Overall, toxicity and systemic absorption of microfine metal oxides are of limited concern.

In light of some of the issues of toxogenicity, photoallergenicity, and photostability surrounding some sunscreens, advanced technologies have been developed to minimize systemic absorption and chemical interaction between combined filters. For instance, Tinsorb™ M, manufactured by Ciba, is a large-molecule hybrid between organic and inorganic filters, which serves to minimize systemic absorption of the microfine inorganic particles. Sunspheres™ are styrene/acrylate polymers filled with water, which dehydrate when applied to the skin, leaving the increased surface area of the hollow sphere to absorb UVR, increasing the SPF of a sunscreen by 50–70%. Products such as Eusolex UV-Pearls™, take advantage of encapsulation technologies to form sunscreen-filled glass beads, which minimize contact allergy and separate UV filters that would be incompatible with other formulations (Tuchinda *et al.*, 2006; Palm and O'Donoghue, 2007).

Aside from human toxicity, concerns have been raised over the effects of sunscreen ingredients on the environment. Sunscreens are relatively stable and enter the environment by washing off of the skin through perspiration, bathing, and recreation. They can pollute the surrounding environment (Fent, Kunz, and Gomez, 2008). UV filters common in sunscreens have also been found in both raw and treated wastewaters in studies conducted in Switzerland and southern California (Balmer *et al.*, 2005; Loraine and Pettigrove, 2005). When these sunscreens enter aquatic environments they can adversely affect the wildlife (Kunz, Galicia, and Fent, 2006). Metal oxides, in particular ZnO nanoparticles, also have adverse effects on marine ecosystems. ZnO nanoparticles can be quite toxic towards algae through their interaction with dissolved Zn^{2+} ions (Wong *et al.*, 2010). Furthermore, some of the currently available UV filters have been specifically associated with coral bleaching, through the release of coral symbiotic zooxanthallae. These sunscreens apparently promote the induction of lysogenic viruses, resulting in the loss of symbiotic zooxanthellae and subsequent bleaching (Danovaro *et al.*, 2008).

8.4 Cyanobacterial secondary metabolites as alternative photoprotectants

8.4.1 Cyanobacterial sunscreens

Given the problems associated with currently available sunscreens, it is medically and environmentally relevant to identify a sunscreen with high efficacy, photostability, and low toxicity, which is also eco-friendly. Many natural products are also environmentally friendly, and thus a sunscreen that fits all of these criteria may very likely be a natural product as opposed to the currently available abiotic, synthetic sunscreens. Some natural sunscreens have been derived from botanical sources, although their effectiveness tends to be associated with their antioxidant properties rather than their ability to absorb UVR. Over 100 botanical extracts have been evaluated and it was found that even among those that could absorb UVR, their extinction coefficients could not compare with their synthetic counterparts and their absorption maxima tended to be in the UVC range (Bobin, Raymond, and Martini, 1994). Some of these candidates include the food colorant annatto, rice bran oil extract gamma oryzanol, licorice, seed oil from the evergreen calyphylum inophylum, and the karanja tree extract pongamol (Kapsner *et al.*, 2005).

Microbial sunscreens also constitute plausible improved alternatives to synthetic or botanical UV filters, and some of them are in fact subjects of patents for that purpose (Gerwick *et al.*, 1995; Jacobs *et al.*, 2002; de la Coba, Aguilera and Figueroa, 2007). In order to screen for potential novel agents, Garcia-Pichel, Wingard, and Castenholz (1993) gave the following conditions: a natural sunscreen should be inducible by UVR exposure, be deposited in a manner optimal for screening (i.e., tegumentary layers), confer a biological benefit (i.e., increased growth or survival rates), and be present under physiological inactivity (i.e., passive defense mechanism). Additionally, a biologically-relevant sunscreen should be photostable and, when removed, should result in the loss of protection. As such, the sunscreens scytonemin and mycosporines discussed above are the most promising candidates derived from cyanobacteria, although other microbial sunscreens may also be appropriate (Gao and Garcia-Pichel, 2011b).

Mycosporines extracted from the red alga *Porphyra umbilicalis* (commonly known as nori) are currently available in the sunscreen product Helioguard 365™ by the Swiss company Mibelle AG Biochemistry. In this product mycosporines absorbing maximally at 335 nm are encapsulated into liposomes, where they have been shown to be photostable and photoprotective *in vitro*. Furthermore, Helioguard 365™, with a mycosporine concentration of 0.005%, reduced lipid peroxidation on human skin and improved skin firmness and smoothness, supporting its ability to protect against premature skin-aging (Schmid, Schürch, and Zülli, 2006). Another product, Helionori™, made by the French company Biosil Technologies, is also composed of mycosporines from *Porphyra umbilicalis*. Helionori™ contains the mycosporines palythine, porphyra 334, and shinorine for maximal absorption at 330 nm. It has also been shown to be photostable and heat-stable, with the ability to reduce sunburn and preserve membrane lipids when used at a concentrations of 1–4%. Furthermore, it is not phototoxic and induces low skin irritability (IC50 > 10%; Cardozo *et al.*, 2007). The possibility of developing a "sunscreen pill", given that mycosporines ingested in the diet can preferentially accumulate in marine invertebrates, has been entertained by some (Karentz *et al.*, 1991; Sommaruga and Garcia-Pichel, 1999; Reynolds, 2011). While none of these products are directly extracted from cyanobacterial sources, the three major MAAs in the nori extracts are found in identical form among the cyanobacteria. An alternative production line based on cyanobacteria would represent perhaps a more sustainable source, which would not compete with edible biomass and would open the door to the development of hyper-producing strains.

The cyanobacterial UVA sunscreen scytonemin has not yet been assessed for human use, although it has great potential given some of its inherent properties such as solubility, photostability, and effective UVA absorption (Gao and Garcia-Pichel, 2011b), all of which have been discussed above (see Table 8.2). In addition to serving as a sunscreen, scytonemin also has anti-inflammatory (Gerwick *et al.*, 1995; Stevenson *et al.*, 2002a) and anti-proliferative properties (Stevenson *et al.*, 2002b; Duan *et al.*, 2010; Zhang, Zhang, and

Kong, 2011). These ancillary biological properties of scytonemin will be discussed in a later section.

8.4.2 Antioxidants

Oxidative stress can result in long-term effects at the cell and tissue levels, often on top of the photoaging syndrome of the human skin, which is characterized by abnormal pigmentation, wrinkle formation, and dryness. Cellular and tissue processes that lead to the symptoms include the induction of metalloproteases that degrade collagen, blood vessel dilation, increased dermal inflammation, irregular keratinocytes, abnormal distribution of melanocytes, a decrease in Langerhans cells, and an amorphous elastin. The hyperpigmentation may result from increased tyrosinase activity, a key enzyme in melanin production, as well as the stimulation of melanogenesis through DNA damage (Fisher *et al.*, 1996; Kawaguchi *et al.*, 1997; Kadono *et al.*, 2001; Pinnell, 2003). Furthermore, singlet oxygen and hydroxyl radicals can damage cells by peroxidation of the cell membrane lipids (Pinnell, 2003; Chaundhuri, 2005). To cope with ROS, cells utilize both enzymatic and non-enzymatic antioxidant responses, which essentially scavenge the ROS generated through UVR exposure. Antioxidants can also increase levels of the tumor suppressor protein p53, which has been shown to initiate apoptosis in precarcinogenic cells (Havre *et al.*, 2002). Through these mechanisms, antioxidants can minimize interactions between ROS and cellular targets and protect the cells from further damage.

8.4.2.1 Naturally-occurring antioxidants

Besides the common antioxidant enzymes superoxide dismutase, catalase, and glutathione-reactive peroxidase and reductase (Thiele, Dreher, and Packer, 2000), many non-enzymatic low molecular weight antioxidants exist (Table 8.3). Those commonly found on the human skin include vitamin C (L-ascorbic acid), vitamin E (α-tocopherol), glutathione, and ubiquinone (coenzyme Q_{10}). Vitamin C is acquired through dietary sources and serves as a major reductant in aqueous environments, where it functions by scavenging free-radicals. It is essential for collagen biosynthesis. Topical vitamin C has also been shown to protect against UVA/UVB-induced erythema in porcine skin and UVB-induced immunosuppression in mice (Pinnell, 2003). In humans, vitamin E is also acquired through dietary sources and is the major antioxidant in the lipid phase. In fact, one of the main functions of vitamin E is to reduce lipid peroxidation in cell membranes by scavenging peroxyl radicals and it may also protect cells by quenching $^1O_2$ (Wang, Balagula, and Osterwalder, 2010). Ubiquinone is synthesized by humans and functions as a lipophilic energy-transfer molecule. Ubiquinone can be found in the skin, where it can serve as an antioxidant by protecting primary human keratinocytes and dermal fibroblasts from UVA-induced oxidations (Shindo *et al.*, 1994; Hoppe *et al.*, 1999).

Plants can serve as a rich source of dietary antioxidant compounds. These include several flavonoids (i.e., silymarin and ferulic acids), pycnogenol, resveratrol, and the tea polyphenols. Silymarin, for example, contains three different flavonoids and acts as an antioxidant through the prevention of lipid

Table 8.3 Common antioxidants and their sources.

Antioxidant compounds	Source
Flavonoids	
• Genistein	Soy, red clover, ginkgo biloba
• Equol	Metabolite of daidzein
• Apigenin	Fruits and leafy vegetables, tea, wine
• Daidzein	Soybeans and plant, red clover
• Silymarin/silibinin	Milk thistle/bioactive component of silymarin
• Caffeic, ferulic acids	Vegetables, olive, olive oil
Polypodium leucotomos	Tropical fern plant *Polypodium leucotomos*
Pycnogenol	Extract from the bark of the maritime pine tree
Resveratrol	Skin and seeds of grapes, nuts, fruits, red wine
Vitamin C (L-ascorbic acid)	Most fruits and vegetables
Vitamin E (α-tocopherol)	Plant oils
Green tea polyphenols	Polyphenolic fractions isolated from tea
• epigallocatechin-3-gallate, epicatechin-3-gallate, epigallocatechin, epicatechin	

Adapted from Wang, S.Q., Balagula, Y., and Osterwalder, U. (2010) Photoprotection: a review of the current and future technologies. Dermatologic Therapies, 23, 31–47.

peroxidation and ROS scavenging (Wang, Balagula, and Osterwalder, 2010). It has also been shown to prevent UVB-induced pyrimidine dimers and hydrogen peroxide-induced DNA damage (Pinnell, 2003). Another example, the tea polyphenols, can constitute up to 40% of the dry weight of the tea leaf and are largely comprised of catechins, which have been shown to quench singlet oxygen, various radicals, and hydrogen peroxide. These polyphenols can also limit UV-induced lipid peroxidation, UVB-induced pyrimidine dimer formation, and inhibit UVB-induced transcriptional activity associated with AP-1, a transcriptional activator of multiple cellular processes involved in differentiation, proliferation, and apoptosis (Katiyar, Perez, and Mukhtar, 2000; Pinnell, 2003).

8.4.2.2 Cyanobacterial antioxidants

Cyanobacteria also serve as a rich source of antioxidants. Most notably, they are capable of producing the brilliantly colored carotenoids seen in plants as well as the colorless mycosporines. Vitamins A and E also serve to defend the cells against harmful ROS.

In addition to serving as accessory pigments in light harvesting, the antioxidant potential of carotenoids has been shown to provide photoprotection to skin exposed to UVR. For instance, β-carotene can modulate the expression of human keratinocytes by UVA (Wertz *et al.*, 2005), astaxanthin may play a role in minimizing or preventing UV-mediated photooxidation of the skin (Guerin, Huntley, and Olaizola, 2003), while zeaxanthin and lutein can inhibit photooxidative damage to the human retina (Neelam *et al.*, 2005). Many studies have also demonstrated an enhanced immune response by β-carotene, including lymphocyte proliferation (Canfield, Forage, and Valenzuela, 1992). As such, the protein and carotenoid-rich cyanobacterium *Arthrospira* spp. (either *A. maxima* or *A. platensis*), commercialized as "Spirulina", is currently used in the commercial production of carotenoids, specifically β-carotene. As of 2006, suppliers such as Hainan Simai in China, Myanmar Spirulina Factory in Yangon, Myanmar, Earthrise Farms in California, USA, and Cyanotech in Hawaii, USA, among others, were producing over 3000 t dry weight of Spirulina annually (Pulz and Gross, 2004). In addition to being protein and carotenoid-rich, Spirulina also accumulates high concentrations of vitamins A and B_{12}, further promoting its use in human and animal nutrition products (Jassby, 1988), even though the use of cyanobacterial-derived vitamin B_{12} as a human supplement has been called into question (Watanabe, 2007).

The antioxidant activity of the mycosporines has not been studied directly for potential application in humans, as current studies do not uncouple the sunscreen and antioxidant benefits of mycosporine products such as the Helioguard 365™ discussed above (Schmid, Schürch, and Zülli, 2006). Even so, the *in vitro* protective effect of mycosporines, specifically mycosporine-glycine, in corals, zooxanthallae, and *Escherichia coli* has been well-documented (Dunlap and Yamamoto, 1995; Suh, Lee, and Jung, 2003).

8.5 Non-photoprotective medicinal applications of scytonemin

Some cyanobacterial photoprotective compounds have potential in medical and industrial applications beyond photoprotection itself. Scytonemin, in particular, is an attractive natural product that has antiproliferative properties. Early studies determined that it was an effective inhibitor of polo-kinase 1 (PLK1), a serine/threonine kinase that functions by phosphorylating CDC25C, a regulator of the G2/M cell cycle transition that is overexpressed in many tumor cells (Stevenson *et al.*, 2002b; McInnes, Mezna, and Fischer, 2005). Even though the functional role of PLK1 in melanoma is not well understood, it has been demonstrated that PLK1 is critical for the survival of melanoma cells (Schmit *et al.*, 2009). Scytonemin shows time- and dose-dependent activity with an IC50 of 2 μM against the recombinant enzyme, minimal cytotoxicity to nonproliferating human monocytes, but lacks specificity for PLK1 (Stevenson *et al.*, 2002b). Duan and co-authors demonstrated that scytonemin slows the growth rate of human osteosarcoma cells and decreases CDC25C protein expression in a dose-dependent manner. Moreover, osteosarcoma cells exhibit a greater sensitivity to scytonemin than normal osteoblast cell lines (Duan *et al.*, 2010). To take this one step further, the cell cycle was monitored in human bladder cancer cells treated with scytonemin. The authors found that not only

did increasing concentrations of scytonemin decrease bladder cancer cell proliferation, but also that it specifically arrests these cells in the G2/M stage of the cell cycle (Zhang, Zhang, and Kong, 2011).

Scytonemin can also act as a powerful anti-inflammatory agent. Since neovascularization is common in inflammatory disorders, human umbilical vein endothelial cell (HUVEC) proliferation was assessed against treatment with scytonemin. With an IC50 of 5.4 μM scytonemin, HUVEC cell proliferation was inhibited and the authors suggested that similar tissue repair cells such as fibroblast and epithelial cells could experience similar effects. Furthermore, they were also able to reduce edema in the mouse ear through topical application of scytonemin (Stevenson *et al.*, 2002a).

8.6 Applications of cyanobacterial sunscreens beyond biomedicine

It seems as though the industrial application of cyanobacterial photoprotective pigments should be relevant as the search for better cosmetics and textiles continues. The cosmetic industry, for example, provides ample opportunity for the development of products with better coverage, protection, and appeal. Many colored cosmetics contain a modest amount of UV filtering agents, primarily in the form of the metal oxides TiO_2 and ZnO, to provide intrinsic SPF protection, albeit in the range of only 2–6 (Draelos, 2009). Facial moisturizers are also widely used and are now commonly found with supplemental sunscreen products. Although not recommended for water-intensive recreational activities, they are most effective for daywear protection. Many of the moisturizers that claim to have anti-aging properties, do so presumably or explicitly on the basis of the active sunscreen ingredient they contain (Idson, 1982). Furthermore, supplementing these products with antioxidants from teas, herbs, and popular fruit products is becoming more common. Thus it is easy to envision how products containing scytonemin or carotenoids, despite their pigmentation, would serve well in the cosmetic industry as sunscreens or antioxidants, respectively. Furthermore, their fat-soluble nature would allow them to easily blend with oil-based foundations, water-in-oil moisturizers, anhydrous creams, or waxes, many of which tend to have a higher SPF and are waterproof. The colorless mycosporines, on the other hand, would offer an "all-in-one" product by protecting against UVB and serving as an antioxidant when added to water-soluble formulations, especially in products marketed for oily and acne-prone skin types, which are designed to be oil-free (Draelos, 2009).

While clothing can provide sun protection simply through coverage, it is estimated that approximately one third of summer clothing may not be effective against UVR and only 75% of fabrics actually impart sufficient protection (Gambichler *et al.*, 2001). In an effort to improve photoprotection in clothing the ultraviolet protection factor (UPF) was developed in Australia in 1996 as a means to measure sun protection efficacy (Gies *et al.*, 1994). Even though polyester, wool, and thicker, darker fabrics have greater UPF values, washing fabrics with optical whitening or UV light-absorbing compounds can improve the UPF of these and other fabrics. For instance, the broad spectrum UVR filter Tinosorb™, manufactured by Rit Sun Guard, can increase the UPF of cotton T-shirts to 30, and remains stable through multiple washings. Similarly, titanium hydrosol and fluorescent whitening agents have also improved the UPF of cotton (Kalia and Haiducu, 2012). One can imagine how detergents could be supplemented with a colorless sunscreen such as the mycosporines or ad hoc derivatives with improved resistance to washout. Scytonemin, on the other hand, is lipid-soluble and should withstand washing, but would likely color the clothing with a yellowish-brown hue, although it may serve well in darker fabrics.

8.7 Translational development: regulatory and commercial aspects

In order to market natural products, regulatory aspects must be considered. The safety of the product must meet specific guidelines and the efficacy must be

subjected to a series of standards to validate manufacturer claims. All of these vary by country and region. In the US, sunscreens are classified as over-the-counter (OTC) drugs, some of which are exempt from governmental preapproval. Depending on the claim and ingredients, some are still subject to an approval process before commercialization. Each governing body has developed a list of preapproved UV filters and methods for determining efficacy and SPF (Steinberg, 2005). As of 2003 there were only 16 approved UV sunscreen active ingredients in the US market, most of which are effective in the UVB range. This is only about one third of all UV filters permitted in the US, EU, Japan, and Australia combined. The maximal content in formulations is also regulated. In the US, the metal oxides are permitted at the highest concentration of 25% (for each of TiO_2 and ZnO) while only 3% is allowed for avobenzone, the most popular UVA filter. There is no standardization among countries regarding maximum allowable concentrations. For example, avobenzone can be used at concentrations of up to 10% in Japan. Permitting procedures are also country specific. In the US, sunscreens can now be marketed through an OTC drug monograph system, which requires neither FDA approval nor a patent protection for the active ingredients.

Commercial development of any product must consider existing patents. Currently, several patents apply to photoprotective compounds from natural sources, including those based on scytonemin (Gerwick *et al.*, 1995; Jacobs *et al.*, 2002) and the mycosporines (De la Coba, Aguilera, and Figueroa, 2007).

8.7.1 Testing standards

To assess UVB filtering efficacy, the standard test is determination of the SPF of the proposed sunscreen product against a standard formulation. Methods for SPF testing were approved in the 1990s by the US, EU, Australia, and Japan, and require *in vivo* tests for redness on an individual's back following topical application of the proposed sunscreen, although there is some variability in the exact method by region. For UVA filtering efficacy, many regions require *in vivo* tests with an artificial UVA source to measure the protection factor of UVA as a function of the minimal persistent pigment-darkening dose of the proposed product on human volunteers (Steinberg, 2005).

8.7.2 Towards production and commercial development

One of the greatest obstacles to scytonemin, mycosporines, and carotenoids becoming commodities and serving a large consumer market is obtaining an increase in biomass production. One approach would be to harvest the product from its native source. Although unclear, this seems to be the method used by Helioguard 365™ in the extraction of mycosporines from *Porphyra*, which involves a heated extraction in ethanol followed by ultra-filtration, a time- and energy-intensive process (Schmid, Schürch, and Zülli, 2006). Furthermore, environmental sources introduce a host of other contaminants, both biological and abiotic. As such, efforts towards optimizing the mass production of cyanobacteria and microalgae are of particular interest and have been widely explored in the biofuel community. Approaches include both open and closed bioreactor systems, which take advantage of the photosynthetic capabilities of these organisms for biomass production through exposure to artificial lighting or sunlight. While open systems such as raceway ponds are cheaper to maintain and do not require as much energy input, closed photobioreactors such as tubes or columns provide more even illumination with less risk from contamination (Brennan and Owende, 2010). In fact, biomass productivity is often higher in closed systems, where strains of *Arthrospira* can reach up to 6 g/l versus less than 2 g/l in open-pond systems (Pushparaj *et al.*, 1997; Carlozzi, 2003). Still others have been successful in growing cyanobacteria heterotrophically for the production of biofuels and other metabolites such as the carotenoids and vitamins mentioned above. Heterologous production of sunscreen compounds from cyanobacteria in bioreactor-friendly microbial hosts such as *Escherichia coli* is possible (Gao and Garcia-Pichel, 2011a), even though it has not been scaled up. While these systems require exogenously supplied carbon and more energy input, they offer more control and lower harvesting costs over light-dependent systems (Chen, Zhang, and Guo, 1996).

As an alternative to total biomass accumulation, efforts could be directed towards increasing the yield

of carotenoid or sunscreen produced per cell. For scytonemin, in particular, which is only produced in response to UVA and only after a lag period of 3–4 days of exposure and high light acclimation, these efforts are not trivial. Furthermore, the cell ceases biosynthesis once it has accumulated sufficient scytonemin to cover the surface area of the cell (Garcia-Pichel and Castenholz, 1991; Gao and Garcia-Pichel, 2011b).

An alternative approach to overcoming these obstacles to mass sunscreen production would be to exert transcriptional control over sunscreen biosynthesis, regardless of the UVR input or the amount of sunscreen already accumulated. This would greatly enhance its potential for mass production and application as a commercial product. Similar methods are used in the antibiotic industry, for example, where the tight transcriptional control of antibiotic production and heterologous expression by non-native host microorganisms is used to produce large quantities of a specific antibiotic (Martin and Gil, 1984). Another approach would be to engineer an export system for the release of mycosporines into solution as is done by the biofuel industry on candidate biodiesel algal and cyanobacterial strains (Liu and Curtiss, 2009).

Even if cultivation or genetic systems could overcome these obstacles to mass production, further problems would persist. Harvesting of the biomass for downstream purification of the metabolite can account for 20–30% of the total production costs (Gudin and Thepenier, 1986). Depending on the properties of the strain and cultivation system, harvesting can be done in bulk through flocculation, flotation, or sedimentation, or by thickening through centrifugation, filtration, or ultrasonic aggregation, all of which offer their own advantages and disadvantages and affect energy-use and costs. For a review of these technologies, see Brennan and Owende (2010). Once the biomass has been harvested the metabolite of interest can then be purified. Some of these approaches affect cell membrane permeability. Techniques for β-carotene purification, for example, use solvents that alter the cell membrane and promote the movement of metabolites towards the outside of the cell. This all depends, of course, on the properties of the cell membrane as well as the chemistry and concentration of the metabolite in the cell (Molina Grima *et al.*, 2003; Hejazi and Wijffels, 2004). Routine extractions of mycosporines and scytonemin in the laboratory, however, require only modest agitation in the presence of methanol or acetone, respectively, and both of these are relatively inexpensive solvents. One could also avoid purification altogether through whole-cell photoprotection. For instance, a dehydrated or dead cyanobacterial cell covered with the stable sunscreen could be introduced into a formulation without the need for pigment extraction. This is similar to the particulate nature of oxide-based sunblock, and where, in addition to absorption, one would take advantage of a light-scattering effect. This approach, however, may add another level of complexity regarding secondary effects and safety regulations.

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Chapter 9

Cyanobacteria as a "green" option for sustainable agriculture

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9.1 Introduction

Cyanobacteria are a morphologically diverse and phylogenetically coherent group of oxygenic phototrophs that successfully colonize and inhabit almost any illuminated habitat. Apart from various natural and man-made aquatic and terrestrial systems, they dominate in many extreme habitats (Sharma *et al.*, 2011). They constitute a substantial portion of soil microbial flora of different agro-ecosystems (a man-made ecosystem) (Prasanna, Jaiswal, and Kaushik, 2008a). Besides the free-living forms, cyanobacteria have developed symbiotic associations with other plants, ranging from algae, bryophytes, pteridophytes, and gymnosperms to angiosperms (Rai, Bergman, and Rasmussen, 2002).

Globally, rice and wheat occupy 75% of the total cultivated area under food grains, and are grown with heavy input of the chemical fertilizers. The widespread application of single (e.g., nitrogen in South East Asian countries) and multiple element fertilizers has led to the accelerated exhaustion of other nutrients, resulting in nutrient imbalance and poor soil fertility. Due to their ability to adapt in diverse environments, cyanobacteria have emerged as a suitable option for restoring soil fertility as well as enhancing plant growth and productivity. Their use as biofertilizer can substantially curtail the use of synthetic fertilizers. Microbial biomass (including cyanobacteria) plays a dual role, as a labile sink and a source of nutrients, in submerged rice systems (Inubushi *et al.*, 1997a, b; Prasanna *et al.*, 2010b). Roger (1996) noted that natural wetland rice systems can produce 2–4 t/ha of grain yield without any fertilizer input.

Enhancement in crop productivity due to the enrichment of soil with selected cyanobacterial strains is known as "algalization." This has resulted in the

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promotion of cyanobacteria-based biofertilizers among the farming community in South-East Asia (Venkataraman, 1972). This article discusses different aspects of the use of cyanobacterial inoculants to enhance crop productivity and improve/sustain soil fertility, especially in the rice–wheat cropping system.

9.2 Diversity of soil cyanobacteria

9.2.1 *Free-living forms*

There are number of studies on the diversity and abundance of cyanobacteria in the rhizosphere of different rice varieties and other crops (Prasanna *et al.*, 2002, 2009a; Ahmed, Stal, and Hasnain, 2010). It was De (1939) who pointed out that the sustaining of fertility of rice fields is due to cyanobacteria. They perform many roles in the soil ecosystem (Figure 9.1):

- fixation of nitrogen and carbon
- promotion of the release of nutrients
- enhancement of the availability of phosphorous
- release of growth promoting auxins, secretion of mucilage/polysaccharides that improve soil structure/porosity
- reduction of the rate of water loss and soil erosion.

Many of the diazotrophic (nitrogen-fixing) cyanobacteria have specialized cells that act as sites of nitrogen fixation. They maintain anaerobic conditions, as oxygen inhibits the activity of the enzyme nitrogenase, which is responsible for nitrogen fixation. The heterocystous and filamentous/colonial forms (microaerobic/anaerobic non-heterocystous nitrogen fixers) constitute the common flora of arable lands. Some of the common filamentous heterocystous forms are *Anabaena*, *Anabaenospsis*, *Aulosira*, *Calothrix*, *Cylindrospermum*, *Fischerella*, *Haplosiphon*, *Mastigocladus*, *Nostoc*, *Scytonema*, *Scytonematopsis*, *Stigonema*, *Tolypothrix*, *Westiella*, and *Westiellopsis* (Stewart *et al.*, 1979; Prasanna and Kaushik, 1995). Amongst the unicellular or colonial forms *Aphanothece*, *Chroococcidiopsis*, *Cyanothece*, *Dermocarpa*, *Gloeothece*, *Myxosarcina*, *Synechococcus*, *Synechocystis*, and *Xenococcus* are common (Rippka *et al.*, 1979;

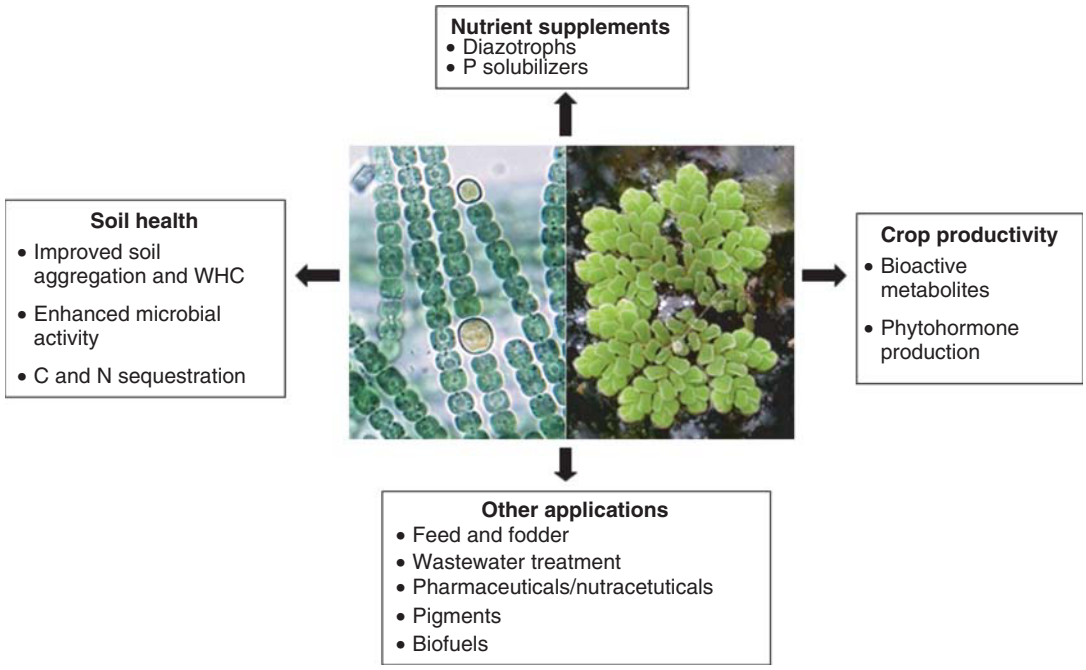

Figure 9.1 Overview of cyanobacteria and *Azolla* applications. See plate section for color version.

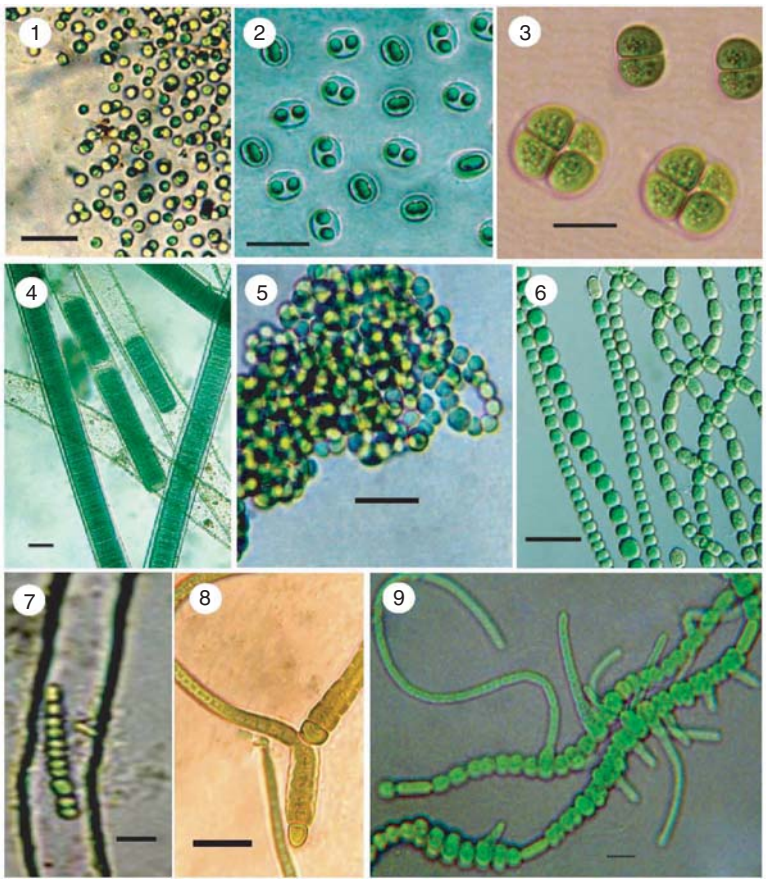

Figure 9.2 Microphotographs of cyanobacteria isolated from rice and wheat fields. (1) *Aphanocapsa* sp.; (2) *Gloeocapsa fusco-lutea*, (3) *Chroococcus turgidus* var. *maximus*, (4) *Lyngbya magnifica*, (5) *Nostoc commune*, (6) *Anabaena* sp., (7) filament of *Anabaena* sp. inside the wheat roots, (8) *Calothix parietina*, (9) *Westiellopsis prolifica* (Bar indicates 20 μm in photographs 1–3, 6–9; 10 μm in 5, and 50 μm in 4). See plate section for color version.

Prasanna and Kaushik, 1994). Non-heterocystous filamentous forms capable of nitrogen fixation are species of *Oscillatoria*, *Trichodesmium*, *Microcoleus*, *Pseudanabaena*, *Plectonema*, and *Lyngbya* (Figure 9.2; Wyatt and Silvey, 1969; Stal and Krumbein, 1985).

Because of their nitrogen fixing abilities, heterocystous filamentous forms belonging to the orders Nostocales and Stigonematales are considered well suited to be biofertilizers. R.N. Singh (1961) made a detailed study of the periodicity of cyanobacterial species in the rice fields of Uttar Pradesh and Bihar states in India, and reported the abundance of *Aulosira fertilissima*, *Anabaena ambigua*, *A. fertilissima*, and *Cylindrospermum*. However, contrary to common belief, nitrogen-fixing cyanobacteria are invariably absent in tropical rice soils. An all-India survey showed that out of 2213 soil samples from rice fields, only about 33% had nitrogen fixers present (Venkataraman, 1979; 1981).

The distribution of cyanobacteria in the soil depends on the soil pH, electrical conductivity, and exchangeable sodium. Amongst these, pH is the most important factor determining the cyanobacterial diversity. Under laboratory conditions, the optimal pH for cyanobacterial growth ranges from 7.5–10, with a lower limit of 6.5–7.0. Although infrequent at pHs below 6.0, cyanobacteria's ability to grow at diverse pH and modify their own environment

make them successful in any niche. There are reports of cyanobacterial presence in soils with pH values between 5 and 6, but in general acidic soils do not support their growth (Aiyer *et al.*, 1971).

A study on the abundance and genera-wise diversity of cyanobacteria in the rice-based cropping systems of north and eastern India revealed the dominance of heterocystous forms (70–90% of the total isolates), with a general pattern of species abundance as follows: *Nostoc* > *Anabaena* > *Phormidium*. The ecology/ecotype of the region and carbon/moisture levels had no effect on the ratio of the dominant cyanobacteria (Prasanna and Nayak, 2007; Nayak and Prasanna, 2007). Moreover, a study of cyanobacterial abundance in rice and wheat pots revealed that inoculants based on two- or three-member cyanobacterial consortia are better than a single species (Prasanna *et al.*, 2009b).

9.3 Role of free-living cyanobacteria in crop growth promotion and productivity

9.3.1 Effect on grain yields

Field experiments performed over the last two decades under the umbrella of the All India Coordinated Research trials used cyanobacterial biofertilizers developed at the Indian Agricultural Research Institute, New Delhi. The study revealed that cyanobacteria can provide 25–30 kg N/ha/season (Venkataraman, 1981), giving an increase of up to 30% in the crop yield (Venkataraman, 1972, Goyal, 1997). Studies of the effect of algalization on grain yield have been carried out in China, Egypt, Japan, the Philippines and India (Singh, R.N. 1961; Jha *et al.*, 1965, Venkataraman and Goyal, 1969; Venkataraman, 1972, 1975, 1978, 1979, 1981; Singh P.K., 1985). The beneficial effects of algalization are attributed to the growth-promoting substances produced by cyanobacteria and the temporary immobilization of added nitrogen, followed by its slow release via cyanobacterial decomposition, resulting in efficient nitrogen utilization by crops (Venkataraman, 1979, 1981).

Comparison of grain yields using different carrier-based biofertilizers vis-à-vis control revealed that biofertilizer based on *multani mitti* (Fuller's earth) gave the highest yield (48.83 q/ha), while the straw- and soil-based biofertilizers gave 46.01 and 44.68 q/ha yields, respectively (Dhar, Prasanna, and Singh, 2007). Rhizospheric cyanobacterial isolates when applied along with a one-third dose of nitrogen and a full dose of phosphorus and potassium fertilizers recorded a 10–20% enhancement in grain weight and 20–30% saving of nitrogen, compared to a full-dose application of chemical fertilizers alone (Prasanna *et al.*, 2011, 2012).

9.3.2 Nitrogen cycling by cyanobacteria

The nitrogen pool in the soil is dynamically maintained through biological nitrogen fixation, the application of fertilizers, and other nitrogen cycling mechanisms (Roger and Ladha, 1992). Nitrogen is brought into the organic farming system through the inclusion of nitrogen-fixing crops in rotation or use of biofertilizers/cyanobacteria in rice crops. Jeffries *et al.* (1992) suggested that soil algae play a major role in nutrient cycling in desert and semi-desert ecosystems, especially in the cycling of nitrogen. As a result, nitrogen-balance studies under such systems are usually positive. A cyanobacterial bloom usually corresponds to less than 10 kg N/ha, while a dense bloom may contain 10–20 kg N/ha (Roger, 1991).

Nitrogen-fixing cyanobacteria readily switch over to other sources of nitrogen (combined nitrogen), if available. However, the majority of the dominant cyanobacteria in rice fields are nitrogen-fixing, which indicates how rice has been grown continuously for many centuries without the addition of fertilizer (Watanabe *et al.*, 1977a, b). The relative contribution of cyanobacteria to the total nitrogen fixed in a paddy field varies widely, and is mainly dependent on chemical, climatic, and biotic factors. In tropical conditions, high humidity and temperature and shade provided by the crop canopy favor the rapid growth of cyanobacteria (Roger and Reynaud, 1979). Furthermore, cyanobacterial inoculation increases the presence of many other useful microbes in the soil. Rao and Burns (1990) observed a several-fold increase in bacterial numbers after 13 weeks of cyanobacterial consortia inoculation, which declined later on (levels were only 2.8 times those at the start after 21 weeks).

Use of *Nostoc muscorum* as an inoculant also produced 500-, 16-, and 48-fold increases in the populations of bacteria, fungi, and actinomycetes, respectively.

Active extracellular release of fixed nitrogen and release upon decomposition are likely sources of nitrogen for higher plants (Jones and Stewart, 1969). It is still unclear whether extracellular products are taken up directly or after microbial degradation. It is reported that only one third of the cyanobacterial population is decomposed and absorbed by the rice crop in the first year, the rest remaining as residual soil nitrogen (Saha and Mandal, 1980). Decomposability of *Anabaena* in soil is reportedly faster than other cyanobacteria, while algal biomass rich in akinetes does not decompose easily compared to vegetative cells (Mandal, Vlek, and Mandal, 1999).

Multilocation trials conducted under varying agroclimatic conditions with different rice varieties indicate that cyanobacterial inoculation can result in the addition of 30 kg N/ha. The biomass of nitrogen-fixing cyanobacteria in rice fields ranges from a few kilograms to about 500 kg dry weight/ha. These cyanobacteria can contribute about 20–30 kg N/ha/season, with the additional advantage of increasing the organic matter content of the soil. The positive effect of cyanobacterial inoculation often increases with time, and only a fraction (2–10%) of nitrogen fixed is immediately available to the crop, the remainder being released following the death and decomposition of the algae. Experiments conducted at the Central Rice Research Institute, Cuttack, indicate that inoculation of soil with *Aulosira* sp. at a rate of 60 kg/ha (fresh weight) causes a significant change in the soil nitrogen content. Cyanobacteria incorporated into the soil increase the nitrogen content by 13–14% under field conditions, and such amended soil releases about 50% of its ammonium nitrogen at 50 days of flooding (Singh, Panigrahi, and Satapathy, 1981). The rates of nitrogen release by the cyanobacteria were 12% and 35% after 7 and 35 days of flooding, respectively (Saha, Panigrahi, and Singh, 1982). Inoculation of the soil with a soil-based mixed culture of four diazotrophic cyanobacteria, namely *Aulosira fertilissima*, *Nostoc muscorum*, *Nostoc commune*, and *Anabaena* species, significantly increased the release of inorganic nitrogen into the soil (Ghosh and Saha, 1997). The nitrogen content of soil inoculated with cyanobacteria was higher when it was light exposed (due to nitrogen gain by cyanobacteria) than that of the unexposed soil (Singh, A.L. and Singh, P.K., 1987b). Chopra and Dube (1971) reported that pots inoculated with *Tolypothrix tenuis* showed a considerable increase in total and organic nitrogen content.

Generally, in wetland rice, recovery of nitrogen fertilizer is 30–40% (in general) with exception rates of 60–65% in rare cases. In such soils, nitrogen is lost rapidly through denitrification (a microbial process), ammonia volatilization (due to a pH increase caused by cyanobacterial and algal activities), leaching, and runoff. Hence management of microbial populations and their activity plays a potential role in wetland rice fields (Roger, Zimmerman, and Lumpkin 1993; Bowen and Rovira, 1999).

Co-cultivation of *Nostoc*/*Anabaena* enhanced the plant nitrogen and root/shoot length (Obreht *et al*., 1993). Studies carried out up to the harvest stage of a crop (Prasanna *et al*., 2009a, b) clearly demonstrated that cyanobacteria enhance plant growth parameters (plant height, dry weight, grain yields) besides bringing about significant changes in soil microbial biomass carbon. Inoculation of *Anabaena variabilis*, *Aulosira fertilissima*, *Nostoc muscorum*, and *Tolypothrix tenuis* individually and/or in combination produces significant improvements in soil microbial biomass carbon and available nitrogen, resulting in improvements in nitrogen uptake and carbon content of the rice plants. The data revealed positive correlations between available nitrogen and soil microbial biomass carbon, and nitrogen uptake and carbon content of the plants (Swarnalakshmi, Dhar, and Singh, 2007a, b). The indigenous population of bacteria, fungi, and actinomycetes in the soil was 500, 16, and 48 times greater, respectively, than that of the non-inoculated soil on day 300 (Rogers and Burns, 1994).

A seasonal change in the microbial community structure may lead to an alteration in the pattern of carbon and nutrient cycling in the soil and vice versa. The positive synergistic effect of biofertilizers and organic amendments (vermicompost and farmyard manure) on soil fertility and nitrogen accumulation advocates for its adoption for wheat crops to improve soil fertility (Prasanna *et al*., 2008b).

A modified technique for the measurement of nitrogen fixation by free cyanobacteria and *Azolla*-cyanobacteria symbiosis under field conditions has been developed (Prasanna *et al*., 2003), using chemical fertilizers, cyanobacteria, and *Azolla* biofertilizers.

The method involves collection of fresh and moist soil cores (0–30 mm) using a soil auger, incubation with 10% acetylene in airtight glass vials under field conditions for 3 h, and measurement of the ethylene produced using a gas chromatograph. Experiments carried out over a period of three consecutive years revealed that the estimates of nitrogen fixation were comparable with the data of other researchers obtained with soil water columns, sophisticated chambers, and automated sampling devices. Those earlier techniques were tedious and time-consuming (Lee and Watanabe, 1977; Watanabe *et al*., 1977a; Roger and Ladha, 1992). Prasanna *et al*. (2006) developed an easy to use and economically viable microtitre-plate-based method for quantifying cyanobacteria (using the most probable number method) in soil samples. This method can be particularly useful for analyzing large number of field samples.

9.3.3 Carbon cycling through cyanobacterial inoculation

Besides their well-established role in nitrogen supplementation, cyanobacteria can be a key player in carbon sequestration, improving nutrient-use efficiency and crop yields (Watanabe and Yamamoto, 1971; Rao and Burns, 1990). Organic matter or organic carbon is considered a key attribute of fertility since it plays a significant role in the physical, chemical, and biological properties of soil. Studies have revealed that the labile organic carbon fractions of paddy soil are influenced largely by nutrient application (fertilizers/manures) and water regimes (waterlogging or alternate wetting and drying). The complex, soil–plant–microbe interactions can influence the plants' health and productivity in many ways (Prasanna *et al*., 2010b).

A compilation of studies from the International Rice Research Institute (Roger, Zimmerman and Lumpkin, 1993) have indicated that, under favourable conditions, 6–8 t/ha fresh biomass can be made available by the growth of cyanobacteria in rice fields. Mucilage, polysaccharides, and resting stages of cyanobacteria such as akinetes provide less easily decomposable biomass (Mandal, Vlek, and Mandal, 1999). Earlier studies (De and Sulaiman, 1950; Aiyer *et al*., 1971; Sankaram, 1977; Das, Mandal, and Mandal, 1991) have shown a buildup of organic matter due to cyanobacterial inoculation in the soil. Under laboratory conditions, native cyanobacterial flora alone can increase the soil organic carbon content by 0.03% (equivalent to 670 kg/ha) within six months (Kaushik 1994, 2004). Inoculation of halotolerant cyanobacteria to sodic soil results in an addition of 5.3–7.6 t C/ha/cropping season. Prasanna *et al*. (2008b, 2012) showed that NPK levels (at one-third, two-thirds, or full doses) significantly increases microbial biomass carbon over the uninoculated control in all the treatments (single-/two-/three-strain combinations) at mid-crop and harvest stages. This is clearly indicative of a microbial build up in the soil because of cyanobacterial inoculation.

The synergistic action of rhizobacteria and cyanobacteria is recorded as promoting rice yields and saving nitrogen fertilizer (30–40 kg/ha), in addition to having a positive influence on carbon sequestration in terms of enhanced organic carbon and microbial biomass carbon (Prasanna *et al*., 2012).

9.3.4 Role in rice fields

In India alone, rice occupies about 45 million hectares and accounts for the largest share of chemical fertilizer use. Traditionally, rice is grown under continuous submerged conditions, which could have adverse effects on the ecosystem – it leads to a tremendous loss of water through percolation, lowers fertilizer-use efficiency, and also has negative impacts on the growth of succeeding crops. In rice-based cropping systems, the role of cyanobacteria in the sustained fertility of flooded/irrigated rice-field soils is well-established (Singh R.N, 1961; Venkataraman, 1979; Roger, 1996). Many free-living strains of nitrogen-fixing cyanobacteria have been isolated and used in biofertilizer consortia in South-East Asian countries, as they do not compete with either crops or the soil's heterotrophic micro-flora for carbon or energy. The potential impact of these organisms on agriculture through their use as biofertilizers, soil conditioners, plant-growth regulators and soil-health ameliorators is been well-recognized (Venkataraman, 1975; Misra and Kaushik, 1989; Vaishampayan *et al*., 2001, Whitton, 2000). It is also well known that cyanobacteria bring about an improvement in the yield of rice (ranging from 5 to 25%), and direct or indirect beneficial

changes in the physical, chemical, and biological properties of the soil and soil–water interface. They enhance the organic carbon, nitrogen, and phosphorus content in the soil, improve soil structure and quality, and produce growth-promoting substances (Kaushik, 1998; Misra and Kaushik, 1989; Nayak *et al.*, 2001, 2004; Venkataraman, 1981) as well as mitigating methane emissions (Prasanna *et al.*, 2002).

In a country such as India, rice is grown in diverse ecologies, in varying soil types, and using different agricultural practices. Exploration of the effects of cyanobacteria under a wide range of conditions is a less-investigated area. Hence, studies need to be undertaken to strengthen the database of cyanobacteria and their functional capacities as a prelude to their use in agriculture.

9.3.5 Cyanobacteria and wheat

Wheat is an agriculturally important crop and requires high doses of fertilizers. However, little work has been done on the supplementary input of cyanobacteria as biofertilizers to this crop, and available information is scarce. A study of the application of vermicompost, farmyard manure, and biofertilizers (cyanobacteria and *Azotobacter*) in different combinations with chemical fertilizers ($N_{40}P_{30}K_{30}$) had promising results for wheat crops (var. HD 2687). The application of vermicompost in combination with cyanobacteria brings about significant increases in nitrogenase activity, while *Azotobacter* + cyanobacteria ($+N_{40}P_{30}K_{30}$) treatment gives the highest values of chlorophyll. The addition of vermicompost and farmyard manure ($+N_{40}P_{30}K_{30}$) enhances cyanobacterial abundance and diversity. *Nostoc*, *Anabaena*, *Calothrix*, *Oscillatoria*, and *Phormidium* are the dominant genera in wheat-crop soil (Prasanna *et al.*, 2008b).

Cyanobacteria are known to liberate substantial quantities of extracellular nitrogenous compounds into the medium. Physiological attributes of a set of cyanobacterial strains (*Calothrix ghosei*, *Westiellopsis*, *Hapalosiphon intricatus*, and *Nostoc* sp.) isolated from the rhizosphere of wheat (var. HD 2687) were analyzed by Karthikeyan *et al.* (2009). The concentrated culture filtrates of these strains enhance wheat-seed germination percentages, and the radicle and coleoptile lengths. Thin layer chromatography analyses of the filtrates revealed the presence of several amino acids such as histidine, and auxin-like compounds. Co-culturing experiments with selected cyanobacterial strains recorded significant enhancements in plant chlorophyll. Root sections of wheat seedlings co-cultured with *Calothrix ghosei* revealed the presence of short filaments inside the root hairs and cortical region (Karthikeyan *et al.*, 2009). Pot culture experiments using cyanobacterial strains (singly or in combination) with variety HD2687 showed a significant enhancement in microbial biomass carbon. The characteristic parameters (plant height, dry weight, and grain yield) were at par with full doses of NPK, (Karthikeyan *et al.*, 2007). Ahmed and co-workers (2010) showed the invasiveness of non-heterocystous cyanobacteria in attacking the epidermis and extensively colonizing the root surfaces of wheat, mungbean, and pea. Such strains can be promising candidates for developing plant-growth-promoting associations for wheat crops and may serve as model systems for understanding the metabolic interactions of cyanobacteria with host plants.

9.3.6 Colonizing inhospitable habitats and their contributions to amelioration

Cyanobacteria not only grow in highly saline alkaline soils, but improve their physicochemical properties by enriching them with carbon, nitrogen, and available phosphorus (Kaushik 1991, 1994). In India, over 10 million hectares of land (mainly the Indo-Gangetic plains, black cotton soils, and the Indian coast line) is estimated to be affected by salts and is inhospitable to crop growth. The predominant cyanobacterial forms recorded in these soils are species of *Nostoc*, *Calothrix*, *Scytonema*, *Hapalosiphon*, and *Westiellopsis*, which appear tolerant to salt fluctuations. Enrichment of these soils with isolates of the indigenous cyanobacteria may help in ameliorating the land and making it suitable for cultivation. In salt-affected soils of Maharashtra (pH 6.0–7.2), the species of *Nostoc* and *Calothrix* are predominant.

Cyanobacteria have the ability to colonize bare areas of rocks and soil, and play an important role in the revegetation of coastal and Usar soils (sodic soils; Singh R.N. 1961). The reclamation of such soils requires

removal of ions using chemical methods, followed by leaching. The process is expensive and does not result in complete reclamation of the soil. Jaiswal *et al.* (2010) bioameliorated the soil with *Nostoc calcicola*, and found that it left land conducive for the growth of plants and microorganisms. Recent investigations have suggested that fly ash combined with organic amendments, nitrogenous fertilizers, and cyanobacteria could be a useful supplement (Tripathi *et al.*, 2004, 2008).

Cyanobacteria contribute to macro-aggregation and produce resistance to soil erosion. As primary producers, they enrich the soil with organic matter and improve the biological activity (Acea, Prieto-Fernández, and Diz-Cid, 2003). Cyanobacterial exopolysaccharides can bind soil particles (Belnap and Gardner 1993; Malam Issa *et al.*, 2007), besides assisting in their motility and their own protection against environmental conditions (De Winder, Stal, and Mur, 1990; Stal, 1995). Inoculation of soil with cyanobacteria brings about improved aggregation of the top soil (Rao and Burns, 1990; Malam Issa *et al.*, 2007), increased water retention capacity, and ecosystem regeneration. Rogers and Burns (1994) demonstrated that inoculation of a poorly structured silt loam soil with *Nostoc muscorum* leads to an enhancement of the soil's chemical properties, with total carbon increasing by 50–63% and nitrogen by 111–120%. In a laboratory experiment, Acea and co-workers (2003) showed that inoculation of soil with different cyanobacterial strains induces significant microbial proliferation as well as large increases in soil organic carbon and available nutrients. However, the efficacy of the treatment depended on the type of soil.

Soil inoculation with cyanobacteria thus represents a simple and low-cost method to improve the productivity of degraded lands.

9.3.7 Cyanobacteria–PGPR consortia: a novel approach

Interactions among microorganisms plays a central role in carbon and nutrient cycling in plant–soil systems. The seasonal change in microbial community structure may lead to alterations in the patterns of carbon and nutrient cycling in the soil and vice versa. Since rhizospheric isolates (plant-growth-promoting rhizobacteria; PGPR) are better competitors than bacteria isolated from other sources due to their direct linkage with roots, their inclusion in biofertilizer/biocontrol consortia can improve the effectiveness of these inoculants. However, there is no published report available on rhizo-cyanobacteria (isolated from the rhizosphere of rice and wheat plants from diverse agro-ecologies) and rhizobacteria interactions or their effects in rice–wheat cropping systems, or in nutrient management and crop-yield improvement. Pot culture experiments using cyanobacterial strains and their combinations and cyanobacteria–rhizobacteria consortia, performed under control and glasshouse conditions, produced enhancements in wheat-plant height, dry weight, and grain yield (Karthikeyan *et al.*, 2007; Nain *et al.*, 2010; Manjunath *et al.*, 2011).

At the Indian Agricultural Research Institute, a seed germination assay was used to screen promising cyanobacterial and bacterial strains. Biochemical characterization of the selected strains revealed PGPR traits such as indol acetic acid (IAA) production, phosphorus solubilization, ammonia production including enhanced soil microbiological activity (alkaline phosphatase activity and fluorescein diacetate (FDA) hydrolysis), and plant growth/yield (Prasanna *et al.*, 2009a). Field-level evaluation of these strains revealed their agronomic efficiency and utility in integrated nutrient management of a wheat crop, besides their significant role in micronutrient biofortification (Rana *et al.*, 2011, 2012).

Pot experiments with the HD 2687 wheat variety, involving various combinations of a set of three bacterial and cyanobacterial isolates each, along with one third nitrogen and a full dose of phosphorus and potassium fertilizers produced increases in the activities of the dehydrogenase and alkaline phosphatase enzymes, FDA, microbial biomass and plant growth. Electron microscopy of the wheat plant roots revealed the mode of entry and intracellular presence of short filaments and single cells of *Nostoc* sp., highlighting their colonization potential (Prasanna *et al.*, 2009b; Nain *et al.*, 2010). Such consortia with multifaceted abilities are useful for integrated farming practices in sustainable agriculture.

9.4 Technology development and innovations

Major limitations for widespread use of cyanobacterial biofertilizer are the lack of sufficient information on their establishment proficiency in diverse crop soil ecologies and ability to compete with native flora. This emphasizes the need to develop more effective formulations and useful markers for their establishment. Two major approaches have been employed for enhancing the promise of cyanobacterial biofertilizers.

9.5 Cyanobacterial formulations – traditional to modern approaches

The systematic approach towards developing a technology for producing cyanobacterial biofertilizers was initiated at IARI, New Delhi, in the 1970s. A rural-oriented open-air mass multiplication technology was developed. Due to its simplicity and economic benefits, it was a success all over India (Venkataraman, 1972; AICPA, 1981). The major advantage of the technology was its use of soil as a carrier and provision for scale-up according to the user requirement (Kaushik, 1994). However, fluctuations in quality due to its open-air nature and aerial- and soil-based contamination hindered its widespread adoption.

Subsequently, efforts were made to formulate an economically viable soil-free medium, using closed structures for bacterial multiplication and alternate carriers (Prasanna and Kaushik, 1998; Prasanna *et al.*, 1998). A nutrient medium using commercial fertilizers was formulated; this was cheaper than the commercially available medium, and could sustain the growth of and nitrogen fixation by the four cyanobacterial strains (*Anabaena variabilis*, *Nostoc muscorum*, *Tolypothrix tenuis*, *Aulosira fertilissima*) used as inoculum. Polyhouses, polythene-covered pits, or cement tanks were used to avoid aerial contamination. Amongst the various carriers tested, wheat straw appeared the best. Cyanobacteria immobilized on straw after drying can be stored for two years without any significant decrease in viability (Prasanna and Kaushik, 1998; Kaushik and Prasanna, 1998). One kilogram of improved biofertilizer was enough for one hectare, compared to 10 kg of soil-based biofertilizer. Another promising carrier used was *multani mitti* (Fuller's earth) possessing excellent water-holding capacity and large surface area (Goyal, 1997; Goyal *et al.*, 1997; Kaushik, 1998; Prasanna *et al.*, 1998). Field-based evaluation of these biofertilizer formulations exhibited the highest yield for the application of *multani mitti* based biofertilizer along with 90 kg N/ha. However, the maximal increase in the yield (37.97%) over control was with 60 kg N/ha (Figure 9.3). The straw-based and soil-based biofertilizer treatments showed the highest yields at 90 and 120 kg N/ha, respectively (Dhar *et al.*, 2007).

Sugarcane and paper waste solid matrices have also been used for the immobilization of cyanobacteria in combination with rice husks and soil (Kannaiyan, 2000). Shanmugasundaram (1996) developed the biofertilizer inoculum "Cyanostraw" by growing the desired cyanobacterial strains along with the carrier under controlled conditions in polybags, obtaining a product free from soil fungi, protozoans, or contaminating algae. A similar method was used in Egypt, but only the floating cyanobacterial flakes, largely free of soil, were collected and dried. About 250 g/ha of dried cyanobacterial flake was inoculated a week after transplanting rice (Watanabe, 1986). Efforts were taken to utilize coir pith, a highly lignocellulosic waste dumped in huge quantities near coir industries. Its soil-conditioning and high water-retention capacities are well established, and generally used in potting media, especially for horticultural crops. Malliga and co-workers (1996) reported lignolytic activity of cyanobacteria, and the utility of coir pith as an inexpensive carrier for cyanobacteria. Furthermore, the coir pith inoculated with cyanobacteria was employed as a biofertilizer – "Cyanopith" and "Cyanospray" – for different crops, especially sunflowers, and resulted in significant yield enhancements (Bhuvaneswari, Subramaniyan, and Malliga, 2011). Coir-pith-based biofertilizer also enhances the quality of fatty acids in groundnuts (Pazhanivel *et al.*, 2011).

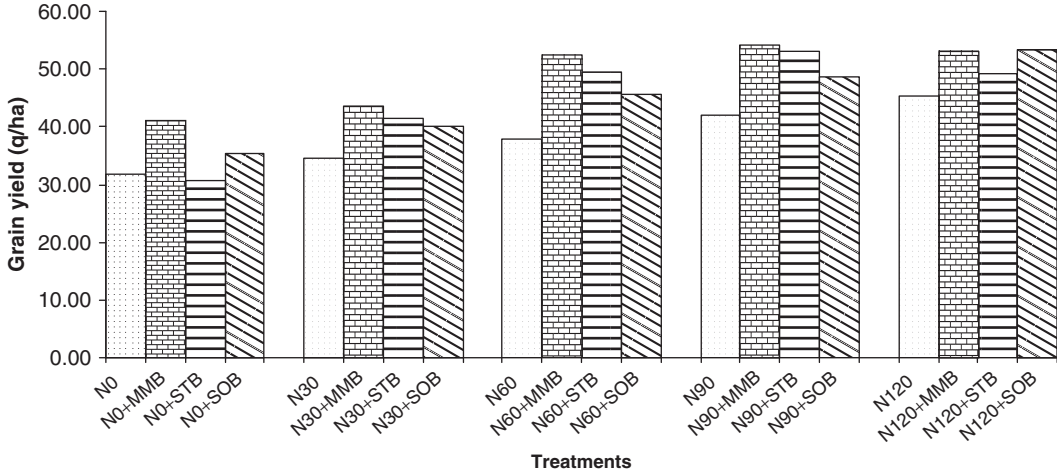

Figure 9.3 Comparative analyses of rice yield as influenced by doses of nitrogenous fertilizer and different carrier-based cyanobacterial biofertilizers (N0, N30, N60, N90, and N120 denote doses, in kg, of nitrogenous fertilizer applied, along with full dose of phosphorus and potassium fertilizers; MMB, SOB, STB denote *Multani mitti*, soil, and straw-based biofertilizers).

Vermicompost used as a carrier for cyanobacteria results in higher soil microbiological and nutritional parameters than fertilizer controls (Prasanna *et al*., 2012). The enhancement in soil microbiological activity and rice plant growth/yield under non-flooded conditions reveals it as an effective carrier. A comparative appraisal of the various carrier-based technologies is provided in Table 9.1.

The presence of predators and grazers in the soil greatly reduces the efficiency of the cyanobacterial inocula (Wilson, Greene, and Alexander, 1980; Grant *et al*., 1983). Snails and nematodes are potential cyanobacterial grazers. However, they differ in their choices for cyanobacterial diet. Snails prefer non-mucilaginous forms, while nematodes prefer mucilaginous forms. Jha and Prasad (2005) explored the application of plant biomass wastes (*Azadirachta indica*, *Aegle marmelos*, and *Nicotiana tabacum*) at 20, 40, 60, and 80 kg/ha as anti-grazer/predator treatments along with cyanobacterial inoculums. The application increased cyanobacterial nitrogenase activity and soil organic carbon, as well as grain and straw yield of rice, accompanied by a decrease in the population of snails and nematodes. There is a need to incorporate such materials in cyanobacterial formulations to encourage their effective establishment in the soil.

9.5.1 Biochemical and molecular markers for monitoring of cyanobacterial establishment in soil

In order to follow the true potential of cyanobacterial inoculants, information on their ability to colonize the roots, their persistence in the soil, and the rhizosphere and saprophytic competence is needed. This in turn, necessitates a need for suitable markers in the inoculated organism(s). The photosynthetic pigments of cyanobacteria tend to mask or restrict the use of *lac Z* or *lux A*, and their use has therefore not been successful (Sankarasubramanian and Kaushik, 2001). Methods based on serology and protein profiles are laborious and time-consuming. Alternative markers need to be screened and feasible methodologies need to be developed.

Prasanna *et al*. (2010a) developed a system for ecological monitoring of cyanobacteria in the soil, using substrate utilization and antibiotic resistance profiles for a set of four strains: *Anabaena torulosa*, *Nostoc carneum*, *Nostoc piscinale*, and *Anabaena doliolum*. These strains were selected from the available germplasm of cyanobacterial isolates from the rhizosphere of rice and wheat. The selection of the strains

Table 9.1 Evaluation of carriers and biofertilizer preparation methodologies.

Parameters	Carrier				
	Soil	**Straw**	**Multani mitti**	**Coir pith**	**Vermicompost/soil: charcoal/compost (1:1)**
Production site	Open air technology, diverse-field, any open area	Indoor using polyhouse/glasshouse	Indoor using polyhouse/glasshouse	Diverse-field, any open area	Indoor using plastic trays
Multiplication units	Shallow polythene lined pits, trays, troughs, bricks/cement lined, RCC tanks	Shallow RCC tanks in one/two tiers	Shallow RCC tanks	Rexin sheets	Plastic trays
Nutrient source	Soil (10 kg/unit +250 g SSP)	Formulated medium	Formulated medium	0.1% neem extract added in the ratio of 1:10 (culture wet weight: dry weight of carrier)	Formulated medium
Energy source	Sunlight	Sunlight or artificial light	Sunlight	Sunlight	Sunlight or artificial light
Aeration	Natural	Can be provided through cloth filters	Can be provided through cloth filters	Natural	Natural
Inoculation	Mixture of strains	Strains grown individually and/or mixed in equal proportions	Strains grown individually and/or mixed in equal proportions	Strains grown individually	Strains grown individually and/or mixed in equal proportions
Carrier qualities	Present everywhere, but contamination cannot be reduced	Easily available, cheap and lightweight	Available in most areas and moderately priced	Cheap and lightweight	Easily available, cheap and lightweight, novel and nutrient enriched
Harvesting cycle	3–4 weeks	7–10 days harvest^{-1}	7–10 days harvest^{-1}	2–3 weeks	2 weeks
Quantity of inoculum required per hectare	10 kg	1 kg	1 kg	250 kg	200 g used as dip for seeds/seedlings to be used for a 36 sq m area
Transportation	Expensive due to bulky nature	Less expensive, light weight	Less expensive, light weight	Site-specific, cheap	Less expensive, light weight
Shelf life	More than 12 months	More than 12 months	More than 12 months	More than 12 months	More than 12 months
References	Venkataraman, 1972,1981	Kaushik, 1994; Prasanna *et al.*, 1998	Goyal *et al.*, 1997	Malliga, Uma and Subramanian, 1996	Nain *et al.*, 2010; Rana *et al.*, 2012; Prasanna *et al.*, 2012a,b

was based on their high nitrogen-fixing potential and persistence in the rhizosphere soil and roots up to the harvest stage in pot experiments. A set of 35 substrates were tested, 53% of which were not utilized by any of the four strains. Citrate, malonate, dextrose, and galactose were utilized by all the strains, while xylose, melibiose, cellobiose, and glucosamine were selectively utilized. All the strains were susceptible to 15 antibiotics. Considering their susceptibility and resistance to different antibiotics tested, unique antibiotic markers were generated (Prasanna *et al*., 2010a). The study aimed to develop methods for quantifying the establishment of inoculated cyanobacteria, using physiological activity (chlorophyll as a growth index and nitrogen-fixing potential as a measure of biofertilizing capacity), along with DNA fingerprints generated using repeat sequences/palindromes, in time course, studies involving microcosm, followed by pot and field experiments. PCR amplification profiles (using STRR 1A, $STRR_{mod}$, and HIP_{AT} sequences) and 16S rRNA gene-based markers proved discriminative in monitoring the presence of the inoculated cyanobacteria in rice- and wheat-crop systems (Prasanna *et al*., 2011, 2012). Based on DNA markers, *Anabaena torulosa* and *Nostoc carneum* exhibited the better persistence among the four strains. Restriction fragment length polymorphism (RFLP) based bioinformatic tool analyses revealed a number of common fragments in the inoculated versus the pure cultures, and these were used to generate a dendrogram. *Alu 1* was found useful for RFLP analyses, as it generated simple patterns with all the samples, and the common fragments could be identified. The dendrogram illustrated the establishment potential of the cyanobacterial strains. Furthermore, despite the lack of flooding/wetland conditions in pot experiments (for the rice crop), the cyanobacterial strains proliferated in the soil. Overall, the cyanobacterial consortia produced 25% nitrogen savings, besides improving the soil fertility and crop-nutritional properties.

9.6 Cyanobacteria in the rhizosphere

The rhizosphere is the hot spot for microbial abundance, diversity, and metabolic activity, and is intimately related to the successful production of crops and the maintenance of soil fertility (Vessey, 2003). However, the rhizosphere of crop plants with respect to cyanobacterial abundance and diversity is less explored. The earliest reports of cyanobacteria in the rhizosphere dates back to the 1980s, when cyanobacterial associations in the rhizospheres of several grasses were investigated (Gantar *et al*., 1991). The majority of the reports have been on cyanobacteria associated with the rice crop, and epiphytic growth of *Gloeotrichia pisum* on the aquatic roots/stem of deep-water rice (Rother *et al*., 1988; Whitton *et al*., 1988).

Studies undertaken to characterize the abundance, diversity, and metabolic capabilities of cyanobacteria isolated from the rice rhizosphere reveal *Anabaena* (40%) and *Nostoc* (38%), followed by *Hapalosiphon*, *Calothrix*, and *Westiellopsis* as the dominant genera. *Anabaena* and *Nostoc* together comprise 80% of the rhizosphere cyanobacterial population (Prasanna *et al*., 2009a). These isolates are also efficient in enhancing the germination and growth of wheat and rice seeds after incubation in light and dark (with 0.5% glucose), and exhibit high protein accumulation and IAA production.

There have been a few attempts to create artificial symbioses (beneficial mutualistic associations) between higher plants and nitrogen-fixing microorganisms (cyanobacteria), for example:

- *Rhizobium*–rice (Al-Mallah, Davey and Cocking, 1989)
- *Anabaena*–tobacco.

Nilsson *et al*. (2002) succeed in establishing artificial associations between cyanobacterial isolates and rice under laboratory conditions. Ahmed and co-workers (2010) described the intimate association of non-heterocystous cyanobacteria with *Triticum aestivum*, *Vigna radiate*, and *Pisum sativum*. This approach could open up a new avenue for improving plant productivity with reduced input of chemical fertilizers.

9.7 *Azolla*–cyanobacteria symbiosis as a biofertilizer

Azolla, a water fern, harbours diazotrophic cyanobacteria and bacteria in its leaf cavities (Wagner, 1997;

Sood, Prasanna, and Singh, 2008a). Its fast growth rate, ability to grow in habitats lacking or having low levels of nitrogen, and in waterlogged conditions has led to its utilization as a mini-biological nitrogen factory in agriculture. It is particularly suited to rice systems (Pabby, Prasanna, and Singh, 2004; Sood and Ahluwalia, 2009).

The *Azolla–Anabaena* association has a long history of use as a green manure for rice crops, and as fodder for poultry and livestock in China and other Far Eastern countries. Its use as a biofertilizer in the paddy fields of the Philippines (Watanabe *et al*., 1977b), India (Singh, P.K., 1977a; Manna and Singh, 1988, 1989a, b, 1990), China (Liu, 1979), Vietnam (Dao and Tran, 1979), Thailand (Loundhapasitiporn and Kanareugesa, 1987), Sri Lanka (Kulasooriya, Hirmburegama, and Abeysekara, 1987), and West Africa (Diara *et al*., 1987) has been well documented. The advantage of integrating *Azolla* into the existing lowland rice-field practices of India is illustrated by Singh (1977b; 1980), who clearly demonstrated its potential as an alternative or supplemental nitrogen source for rice grown in tropical and temperate regions (Table 9.2). It is most effective when grown as a green manure during the fallow season of rice, incorporated in the soil together with nitrogen fertilizer in a dual culture (Singh, P.K. 1979a,b). The annual production of *Azolla pinnata* at farms at the Central Rice Research Institute, Cuttack, India (three years' average) was about 347 t fresh weight/ha, which contained 868 kg nitrogen (Singh, P.K., 1980). Although the agronomic potential of *Azolla* as a biofertilizer is well established, its sensitivity to temperature (Pabby, Ahluwalia, and Dua, 2002a,2002b), plasticity of character (Sood, Prasanna, and Singh, 2007; Kannaiyan and Rains, 1985; Kannaiyan *et al*., 1988) and the disputed nature of the cyanobiont (Sood, Prasanna, and Singh 2008a; Sood *et al*., 2008b) have hindered the application of genetic modification. A lack of knowledge about the factors controlling spore formation and germination, and limited knowledge on generation of hybrids with more acceptable characteristics at field level are other areas requiring attention.

The application of sporocarps instead of fresh *Azolla* biomass to fields can solve many problems, mainly the huge size of the initial inoculum and the consequent difficulties in its transport. Kannaiyan (1990) developed a technology named "Frond-based dried spore inoculum," which involved sporocarp production, collection, storage, and germination. However, its adoption is still in the nascent stage.

There are three primary methods of fresh *Azolla* application to the field. First, it can be grown in the field as a monocrop during the fallow season and then incorporated into the soil before planting the target crop. Secondly, it can be grown as an intercrop along with the target crop. Thirdly, naturally growing *Azolla* can be harvested from ponds, swamps, or flooded fields and applied to a variety of target crops, either before planting the crop or as mulch around the base of crop plants. Often a combination of these methods is utilized. In monocropping, *Azolla* is allowed to grow in the fallow season as a monocrop, and rice plants are transplanted after its decomposition (Singh, P.K. 1977b; Singh, A.L. and Singh, P.K., 1987a,b,c,d). This type of application makes nitrogen available during initial stages of plant growth. *Azolla* is also grown along with the rice crop and incorporated once or twice during the crop growth. This makes nitrogen available during the maturation stage of crop (Misra and Singh, 1988; Singh, P.K. 1988, 1998).

9.7.1 *Integrated cropping system using* Azolla

The mass production and maintenance of *Azolla* is labour intensive, and requires proper care. Therefore, integrated farming systems such as rice–*Azolla*, rice–*Azolla*–fish, rice–duck–*Azolla*, and rice–*Azolla*–fish–duck have been traditionally and commonly practiced in South-East Asia (Tang *et al*., 2000; Zhang *et al*., 2010). Wang and co-workers (2007) have observed that multiple-species coexistence models are effective in controlling *Thanatephorus cucumeris* (Frank) Donk, *Nilaparvata lugens* Stal, *Chaphalocrocis medinalis* Gueneeand, and weeds. The overall weed-inhibiting effect of an integrated rice–fish–duck system is higher than that of the other farming systems such as rice monoculture or rice–fish culture in the traditional agricultural region in Congjiang County of Guizhou Province (Zhang *et al*., 2010).

9.7.1.1 Rice–*Azolla*–fish culture

China has a long history of raising fish in rice fields. However, the yield is low because of difficulties in applying feed to the large areas of fish-raising fields. *Azolla* is an excellent feed for fish (having fast growth, high yield, and a suitable size for fish grazing). *Azolla* is rich in the amino acid arginine, which plays an important role in fish growth. Moreover, the environment created by rice–*Azolla*–fish system is not conducive for the survival of natural enemies of rice pests (e.g., spiders and black ants), thus cutting down pesticide requirements.

9.7.1.2 Rice–duck–*Azolla* culture

The Japanese rear ducks in paddy fields along with *Azolla* plants (Kishida and Utsumiya, 1998; Kishida and Okazaki, 1999). Such practice improves the paddy soil fertility by increasing organic matter (7.95%), the availability of nitrogen (7.05%), phosphorus (6.47%) and potassium (4.46%), and also by controlling weeds (98.94%). It is also effective in controlling plant hoppers, and thus the incidence of rice stripe disease (Zhen *et al*., 2006).

9.7.1.3 Rice–*Azolla*–duck–fish

A study indicates a significant increase of rice yield – 33% in rice–fish–*Azolla* and 25% in rice–fish–duck – of 58% in rice–*Azolla*–duck–fish system. Among the ten rice-based systems, the rice–fish–duck–*Azolla* and rice–*Azolla*–duck systems gave the highest (4 t/ha) rice grain yields (Zhen *et al*., 2006).

9.7.2 Effect of Azolla supplementation on grain yield

A wide variation in the rice grain yield was observed after incorporation of *Azolla* in paddy rice cultivation. The increments reported in the grain yield were 14–40% (Moore, 1969), 25% (Watanabe *et al*., 1977a), 9–38% (Singh, P.K. 1977c), 35.8% (Kannaiyan, 1981) and 13–20% (Watanabe, 1984). This variation could be due to the different experimental methods, soil characteristics, and environmental factors (Table 9.2). In addition, the effectiveness of *Azolla* as biofertilizer varies from species to species and depending on climatic conditions, the method of application (Singh A.L. and Singh P.K., 1990), dosages of various chemical fertilizers, and the time of application (Manna and Singh, 1989a,1989b; Table 9.2).

Application of *Azolla* as a monocrop increases the rice yield by 112% against unfertilized control, by 23% when used as an intercrop, and by 216% when used as both monocrop and intercrop (Peter, 1978). Kondo and coworkers (1989) applied *A. pinnata* as an intercrop by inoculating it 5 days after transplanting the rice, then re-inoculating 27 days after transplanting, and recorded an increase of 27% in the grain yield. Application of *Azolla* as a monocrop and intercrop incorporated 40 days after transplantation gave a 30.6% increase in rice grain yield (Lay, Huang, and Wang, 1989). Singh A.L. and Singh P.K. (1989) recorded grain yields of 5.6 t/ha when 0.5 t/ha of fresh *Azolla* was inoculated 20 days before transplanting and incorporated into the soil after 15 days of growth, followed by another application of 0.5 t/ha fresh *Azolla* 10 or 30 days after transplanting. The grain yield was equal to that achieved with the application of 60 kg N/ha urea (Table 9.2).

A single crop of *Azolla* provides 20–40 kg N/ha (Watanabe *et al*., 1977a). However, this is insufficient to meet the total nitrogen requirement of the crop. Therefore, the use of *Azolla* is recommended in combination with chemical nitrogen fertilizers (Singh P.K., 1982). Singh A.L. and Singh P.K. (1987b,c) also observed that application of 30 kg N/ha urea with *Azolla* (as basal dose) gave grain yield equivalent to that of three split applications of 60 kg N/ha.

The biofertilizer potential of *Azolla* was compared with other biofertilizers. Singh P.K. (1982) observed the highest rice grain yield (5.0 t/ha) with application of *Azolla* +120 kg N/ha, followed by cyanobacteria + *Azolla* + 60 kg N/ha (4.62 t/ha) and 120 kg N/ha (4.61 t/ha). Singh A.L., Singh P.K., and Singh P.L. (1988) observed that *Azolla* and *Eichhornia* compost, farmyard manure, and *Sesbania* green manure produced lower grain yields and panicles than 60 kg nitrogen as urea in the first year. On the other hand, the second-year grain yields produced by all these treatments were equal. In another experiment, the

Table 9.2 Benefits of *Azolla* application to rice.

Treatment	Time of *Azolla* inoculum application	Time and amount of N fertilizer (kg N/ha)	Grain yield (t/ha)			% increase in grain yield against N application	Reference
			Control (no N)	+ N	*Azolla* treatment		
0.5 t *Azolla* FW/ha	20 DBT + 10 and 30 DAT or 30 and 50 DAS	Information not available	3.1	5.6 (60 kg N/ha)	5.6 (dry)	Equal yield as that of chemical N-fertilizer	Singh and Singh, 1989
			2.6	4.2	4.3 (wet)		
Azolla (500 kg FW/ha) + 30 kg N/ha	1 WAT	30 kg N/ha as 50:25:25 before transplanting, maximum tillering, and panicle stage	2.8	3.3	4.0 (dry)	17.5%	Singh and Singh, 1987
			2.7	3.0	3.7 (wet)	18.9%	
Azolla 20 t (basal)/ha + 30 kg N/ha	5 DBT	30 kg N/ha, three split doses before transplanting, 20 DAT, 45 DAT	1.92	2.9 (60 kg N/ha)	3.08 (wet)	5.8%	Singh and Singh, 1987a
			2.83	3.87	4.07 (dry)	4.9%	
90 kg N + 500 kg fresh *Azolla* FW/ha	10 DAT	90 kg N/ha as basal, 20 DAT, 40 DAT	3.2	5.4	6.1 (dry)	11.4%	Singh, Singh and Lata, 1988
			2.4	3.8	4.2 (wet)	9.5%	
90 kg N + 1 t *Azolla* FW/ha	7 DAT	50, 25 and 25% at planting, tillering ,and panicle initiation	2.4	3.6	3.9 (dry)	7.6%	Manna and Singh, 1988
			3.3	3.9	4.3 (wet)	9.3%	
30 kg N + 2 t *Azolla* FW/ha	20 DBT	@ 15 kg N/ha as basal , 50 DAT	3.37	4.82	5.2	7.3%	Singh *et al.*, 1988
40 kg N + 1 t *Azolla*/ha	7 DAT	25:50:25 at 0 d, 30 DAT and 40 d before crop maturity	2.9	3.5	4.23 (dry)	7.3%	Manna and Singh, 1989a
			3.68	4.52	5.54 (wet)	18.4%	
90 kg N + 1 t FW *Azolla*/ha	7 DAT	50:25:25 at planting, tillering, and panicle initiation stage	2.13	3.28	3.65 (dry)	10.1%	Manna and Singh, 1989b
			3.1	4.25	4.46 (wet)	4.7%	

DBT, days before transplanting; DAS, days after sowing; DAT, days after transplanting; WAT, week after transplanting; FW, fresh weight.

incorporation of *Azolla*, once before and twice after transplanting, produced higher grain yields than other organic and green manures (Singh A.L., Singh P.K., and Singh P.L., 1988).

In nature, the growth and nitrogen fixing capacity of *Azolla* is limited by phosphorus availability, which is generally low to meet the requirement of a fern (Subudhi and Singh, 1979; Singh D.P. and Singh P.K., 1988; Sood, Ahluwalia, and Dua, 2005). Therefore, the effect of phosphorous application on growth, nitrogen fixation, and grain yield of the rice has been observed (Singh D.P. and Singh P.K., 1988; 1989; 1995). Singh D.P. and Singh P.K. (1995) reported an increased grain yield and nitrogen uptake by rice plants in response to phosphorus enrichment.

9.7.3 Azolla and soil health

Azolla contains 3–6% nitrogen, 0.5–0.9% phosphorus and 2–4.5% potassium on a dry weight basis, along with other micronutrients (Watanabe *et al*., 1977b). It is well documented that the application of *Azolla* improves soil fertility and productivity of rice crop, rice-green gram, and maize–mustard cropping systems (Singh A.L. and Singh P.K., 1987a,d; Anup *et al*., 2010; Rajarathinam *et al*., 2010). The enhancement is because of increases in the organic carbon, and the available nitrogen and phosphorus in the soil (Singh, Panigrahi, and Satapathy, 1981; Rajarathinam *et al*., 2011). Addition of organic matter improves soil pH, porosity, soil texture, and other physicochemical properties. Ventura and Watanabe (1993) and Awodun (2008) reported that the continuous application of *Azolla* decreases the bulk density of the soil and increases its porosity. The availability of nitrogen and phosphorus to the rice plants varies with species/strain of *Azolla*, nitrogen content, chemical composition, and the time of incubation (Singh Panigrahi, and Satapathy, 1981; Singh Y.V. and Mandal, 2000). Singh and co-workers (1981) reported that *A. pinnata* releases ammoniacal nitrogen more efficiently than *Azolla filiculoides* and *Azolla mexicana*. The release of ammoniacal nitrogen by different organic manures decreases in following order: *Azolla* > farmyard manure > *Sesbania* > cyanobacteria and *Azolla* compost. *Azolla* helps in mineralization of nitrogen and phosphorus from organic materials and chemical fertilizers (Singh and Mandal, 2000).

9.8 Concluding remarks

With the renewed and growing interest in organic farming, the use of indigenous native technologies and environment friendly supplements will form the basis of "green" technologies for environmentally safe and sustainable agriculture. Depletion of soil fertility, low fertilizer-use efficiency, growing environmental pollution, and declining crop productivity are major concerns. In the current scenario, the emphasis in agriculture is to enhance the efficiency of integrated nutrient management systems, promoting the use of low chemical inputs amidst improved nutrient-use efficiency. A better understanding of interactions among microbes, fertilizers, and plants can lead to sustainable integrated nutrient-management practices. The use of cyanobacteria (free-living and symbiotic) may be an effective supplement to organic agricultural practices. Studies of the ecology of cyanobacteria in different and sometimes extreme environments may provide useful information for selection of region-specific inocula that will maximize the utility of cyanobacterial inoculants.

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Chapter 10

The economics of cyanobacteria-based biofuel production: challenges and opportunities

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10.1 Introduction

The contribution of energy to economic growth is a debatable issue, but there is little doubt that it is important for global economic growth (Stern, 2011). A rough estimate suggests that fossil fuels currently support 80% of our energy needs. Between 2002 and 2025, global energy consumption is supposed to increase by 57%, from 435 trillion MJ to 681 trillion MJ (Otero, Panagiotou, and Olsson, 2007). The use of fossil energy has serious economic (high price), ecological (polluting the environment), and sustainability-related (non-renewable, limited future supply) implications. Renewable sources of energy are key to energy security (depleting of oil reserves, high price) and global security (geopolitical dimensions), economic growth, and the stability of the global climate (climate change mitigation) (Stephens *et al*., 2010; Robbins, 2011). With global population reaching more than 7 billion, a sustainable solution to rising food and energy demands warrants a shift towards renewable, eco-friendly and sustainable form of energy (Song, Fu, and Shi, 2008; Sheehan, 2009; Solomon, 2010).

A sustainable energy source should essentially emit less greenhouse gases, has little or no adverse effect on biodiversity (land for biofuel) and global food security, and has a low water footprint (i.e., the total annual volume of fresh water used to produce them)

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(Tilman *et al.*, 2009; Gebens-Leenes, Hoekstra, and van der Meer, 2009). The use of biofuels derived from crop products, wood, organic wastes, and microbial biomass, is one amongst the many renewable energy options available to us (Kopetz, 2013).

According to the International Energy Agency, biofuels are likely to deliver nearly 25% of the world's projected energy needs (623 exajoules) by 2035, with the current share only about 10% (53 exajoules; IEA, 2011, 2012). The bulk of these biofuels is used for heating, electricity generation, and for transportation. A shift to biofuel would not only improve energy security but would help to curb global warming and to create jobs, especially in rural areas (Gilbert, 2011; Kopetz, 2013). Current research on bioenergy is mainly focused on the conversion of biomass into biofuels and biodiesel. Biomass refers to the non-fossilized organic matter (e.g., agricultural crops, forestry products, microbial biomass, and agricultural, industrial, and household organic wastes; FAO 2006; Gebens-Leenes, Hoekstra, and van der Meer, 2009). Theoretically, all crops can be used for bioenergy production, but the emphasis has been on the use of agricultural crops such as sugarcane, corn, sugar beet, rapeseed, soybeans, and jatropha for biofuel production (Gebens-Leenes, Hoekstrah and van der Meer, 2009). Of the total world bioethanol production, 60% comes from sugar crops, 33% from other sources, and 7% from chemical synthesis (Dexter and Fu, 2009). However, the use of crop plants for biofuel production has caused a fair amount of criticism owing to the huge investments required in terms of land, water, energy, and money (Tilman *et al.*, 2009; Martin, 2010). Contrary to the opinion that bioenergy compromises food security, the evidence suggests that it could help to bring food security to the world's poorest (Lynd and Woods, 2011; Kopetz, 2013).

For many years, researchers have tried to use microalgae (green algae, diatoms, and cyanobacteria) as a potential source of biofuels and biodiesel. Under certain conditions, some microalgae accumulate considerable amounts of lipids (more than 50% of their ash-free cell dry weight), which could be used as fungible fuel. Moreover, use of microalgae for biofuel production has many advantages over other plant resources, such as

- high per-acre productivity
- use of non-food-based feedstock resources
- use of otherwise non-productive, non-arable land
- utilization of a wide variety of water sources (fresh, brackish, saline, marine, produced, and wastewater)
- production of both biofuels and valuable co-products (the biorefinery approach)
- sequestration of carbon dioxide
- potential removal of heavy metals and other nutrients from the waste streams.

Plants such as switch grass (the most efficient plant) and corn have energy conversion efficiencies of about 1% and 0.25%, respectively (Masukawa, Mochimaru, and Sakurai, 2002), while algae can capture up to 10% of solar energy as biomass (Niederholtmeyer *et al.*, 2010). The current share of microalgal biofuels in the total biofuel production is around 4%, and is likely to increase in near future. Biofuels are likely to have less impacts on climate change, human health, and ecosystem quality than fossil fuels.

Cyanobacteria are photosynthetic bacteria exhibiting plant-like oxygenic photosynthesis and were present early in the history of the Earth. Because of the simple growth requirements of many cyanobacteria (e.g., the nitrogen-fixing species) and the ease of genetic manipulation in some of them, they have emerged as a viable option for biofuel production (Benemann and Oswald, 1996; Jansson, 2012). Particularly in areas that are normally unsuitable for conventional crops, cyanobacteria may be mass cultivated. Their use for biofuel production vis-à-vis terrestrial plants has many advantages (Table 10.1). The conceptual framework of using cyanobacteria as an alternative and renewable source of biofuels has been available for a long time. However, a scalable, sustainable, and commercially viable system has yet to emerge (Stephens *et al.*, 2010). In the current scenario, biofuels based on algae, including cyanobacteria, are expensive, complex to produce, and are only just entering the commercial phase in small quantities in pilot or demonstration plants. There are subtle challenges to the economic production of cyanobacterial biofuels at commercial scales and it is essential to identify them. In this article, we discuss the current scenario of using cyanobacteria for the production of liquid transportation biofuels. In addition, the feasibility and economic challenges associated with scaling up of the biotechnological processes are discussed.

Table 10.1 Advantages of using cyanobacteria as feedstock vis-à-vis other plant-based biomass sources.

Parameters	Cyanobacteria	Other plant-based biomass resources (e.g., sugarcane, sugar beet, corn, and cellulose-yielding trees)
Land requirement	Require less area, even deserts may be utilized. Closed, controlled indoor photo-bioreactors can be developed anywhere (location independent)	Require a vast area of land to support the global biofuel demand, and may hamper the global food security and escalate the loss of biodiversity
Water requirement	Low water footprint; certain species are capable of growing in saline waters	High freshwater footprint
Energy effectiveness	Energy required to run photo-bioreactors is less	Energy intensive process, e.g., it takes nearly 2 or 3 gallons of oil to produce a gallon of corn-based ethanol
Greenhouse-gas emission	Eco-friendly, release negligible amount of greenhouse-gases (e.g., NO_x released during H_2 production). Cyanobacterial blooms have negative effects on aquatic ecosystems, but seen as a potential alternative strategy to sequester the excess carbon released into the atmosphere	Use of fossil fuel during conversion, and clearing of forests for land may lead to the release of greenhouse gases into the atmosphere
Photosynthetic efficiency	High, and can be further extended through genetic manipulation and use of designed photonic systems (LED techniques) for photo-bioreactors	Low
Global food security	No effect	Serious impact on global food security
Effect of climate change on productivity	Photo-bioreactors are insulated from climatic changes	Productivity of biofuel crops may be severely affected by climatic changes. For example, sugarcane requires tropical climate to grow
Biotechnological manipulation ability	Open, with enormous potential for improvements in growth rate and production level through genetic engineering	Majority are extremely mature, have undergone trait improvements for centuries and are unlikely to be improved much beyond their current production level
Sustainability	Highly sustainable	High ecological footprints; competes directly with global food production, hence low sustainability
Pollution generation	Use simple inorganic nutrients for growth	Use of fertilizers to increase the production will have adverse effect on ecosystem health
Cost effectiveness	Have no adverse effect on food prices, hence the cost of biofuel is likely to be less	Use of crops for biofuels has resulted in the increased global food price. This may also increase the price of biofuels

10.2 Cyanobacteria as a biofuel source

10.2.1 Bioethanol production from cyanobacteria

Figure 10.1 shows the strategies available to utilize cyanobacteria for biofuel production. In biomass-based biofuel production processes, carbohydrates (e.g., sugars and polysaccharides) are fermented into ethanol (first-generation technology), or residual parts such as cellulose (also *de novo* synthesized) are converted into biofuels (second-generation technology). However, these processes are inefficient and constrained by the limited capacity of these organisms to store the substrates intracellularly (Angermayr *et al.*, 2009).

Apart from their simple growth requirements, the possibility of genetic manipulation of some strains

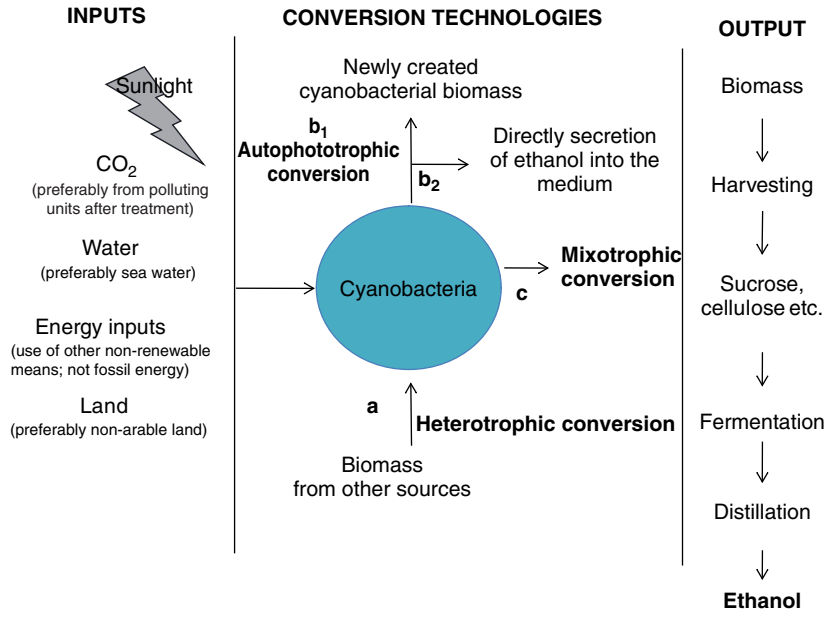

Figure 10.1 Cyanobacterial biofuel conversion technologies. (a) Cyanobacteria can be used to convert biomass from other sources into biofuel (heterotrophic conversion). (b1) In phototrophic conversion cyanobacteria produce biomass, which can be converted into biofuel. (b2) Alternatively, cyanobacteria can be tailored to produce feedstock (e.g., sucrose, cellulose) directly in the growth medium. (c) A new approach is to combine both phototrophic and heterotrophic metabolisms to directly produce bioethanol.

has potentiated the use of cyanobacteria for biofuel production (Nobles, Ramanovicz, and Brown, 2001; Koksharova and Wolk, 2002; Tamagnini *et al.*, 2007; Ananyev, Carrieri, and Dismukes, 2008; Brown and Nobles, 2008; Fu, 2009; Angermayr *et al.*, 2009 and references therein). Brown and Nobles (2008) have designed strains of cyanobacteria that could produce biofuel feedstocks (sugar and cellulose) in excess, and secrete them into the liquid (growth) medium. This avoids any harm to the cells, and facilitates continuous production of feedstock without affecting the harvesting process.

Hellingwerf and Teixeira de Mattos (2009) proposed the "photanol approach" to maximizing the production of biofuels (see Chapter 11). In this approach, a photosynthetic organism is genetically modified in such a way that it converts metabolic intermediates directly into a biofuel product through the addition of a heterologous fermentative metabolic pathway while photosynthesis fuels carbon dioxide fixation (third and fourth-generation technologies; Angermayr *et al.*, 2009; Hellingwerf and Teixeira de Mattos 2009).

Cyanobacteria may also be designed to produce ethanol with high efficiency by the combination of gene transformation, strain/process development, and metabolic modeling/profiling analysis. *Synechococcus* and *Synechocystis* are the best-characterized cyanobacteria for biofuels production. Deng and Coleman (1999) transformed *Synechococcus* sp. PCC 7942 with the pyruvate-decarboxylase and alcohol-dehydrogenase genes of *Zymomonas mobilis* to produce ethanol. Dexter and Fu (2009) successfully transferred the pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase II (*adh*) genes from *Zymomonas mobilis* (a prolific ethanol producer) into *Synechocystis* sp. PCC 6803 using the light-driven psbA2 promoter.

Both extrinsic (light, temperature, salinity, micronutrient, carbon and nitrogen sources, oxygen) and intrinsic factors (the genetic component) play crucial roles in optimizing cyanobacteria-based

feedstock production. Increasing the photosynthetic efficiency could increase cyanobacterial biomass production. Mussgnug *et al.* (2007) increased photosynthetic efficiency by modulating the size of antenna. Adopting advanced photobioreacter technology also enhances cyanobacterial production (Gordon and Polle 2007; Lehr and Posten 2009), particularly the supply of low-energy artificial light optimized for photosynthesis (Park, Kim, and Lee, 2000). However, the biofuel thus produced is costly and cannot compete with fossil fuels.

Researchers such as Walker (2009) think that microalgae – including cyanobacteria – are intrinsically not more but *less* productive than crop plants. He argues that the contribution of algal biofuel to our energy requirements and carbon sequestration will be marginal. Furthermore, ethanol as a biofuel has certain disadvantages. Its high solubility in water necessitates energy-intensive distillation and it has only two-thirds of the energy density of petroleum. Because of the hygroscopic nature of ethanol it is difficult to distribute it through pipelines, and there is an engine incompatibility at higher blending volumes (Otero, Panagiotou, and Olsson, 2007; Savage, 2011). Ethanol is also less suitable as a fuel for aircraft and trucks, which are the big parts of the transportation industry (Service, 2011). Another study suggests that the mere use of biofuels can only marginally improve the city air quality (Service, 2012).

The Dueling Fuels website (2008) estimated that approximately 5000–15,000 gal of ethanol/acre/year (46,760–140,290 l/ha) can be produced from microalgae, which is much higher than the quantity obtained for other commonly used feedstocks (Mussatto *et al.*, 2010). Similarly, Algenol Biofuels Inc. has claimed that its plant is able to produce ethanol at a rate of over 6000 gal/acre/year. With further refinements, the company expects to increase production to 20,000 gal/acre/year in the near future. The strains developed and used by the company are capable of tolerating high temperature, high salinity, and the high alcohol levels present during ethanol production (Algenol Biofuels, 2010). Conversion of cellulose to ethanol has many advantages over sugar-to-ethanol conversion. However, related technologies are still under development and many challenges remain to be overcome (Mussatto *et al.*, 2010).

10.3 Factors affecting the economics of liquid cyanobacterial biofuel production

Although fossil fuels are still among the most cost-effective energy sources today, it is anticipated that their price will rapidly increase as their supply becomes limited. Moreover, fossil fuels have been a source of political instability (Robbins, 2011). The market for transportation fuels is worth trillions of dollars per year, and there is a huge opportunity to replace fossil fuels with biofuels, which already have major market share in the shape of sugar based ethanol (e.g., corn-based) and biodiesel from soybean and oil palm (Service, 2011). The limitations of using crops for biofuels, alongside increased funding and technological advancement for algal fuels, have renewed hope and convinced many of the possibility of developing algal transportation fuels (Mascarelli, 2009; Fairley, 2011; Savage, 2011; Service, 2011; Georgianna and Mayfield, 2012). However, algae-based fuels are expensive. The cheapest algae-based biofuels cost about $2.25/l, which is more than twice today's average gasoline price in the United States (Service, 2011), although comparisons are difficult because of taxes and subsidies. The ways to cut the cost depends on the type of alga used, the type of photobioreactor, and the production technology of the biofuel. In case of biomass production from microalgae, Norsker *et al.* (2011) calculated the cost for three different commercial-scale production systems: open ponds, horizontal tubular photobioreactorss and flat-panel photobioreactors, with respective costs of €4.95, €4.15, and €5.96/kg dry biomass. The major cost factors were irradiation conditions, mixing, photosynthetic efficiency of the algae, and the medium and carbon-dioxide costs. The production cost came down to €0.68/kg (a cost-competitive price) when these factors were optimized. Other parameters important for cost-competitive biofuel production are enhanced energy capturing efficiency, high production rate, and simple inputs.

Growing microalgae in sunny, open-air, shallow ponds is the most popular strategy. However, such systems suffer from self-shading and the algae grow

at a low density, so a large amount of energy is required to harvest them. Closed bioreactors allow algal growth at a much higher densities but are more expensive than open ponds. Other aspects that compromise the efficiency of cultivation systems with a considerable impact on the overall economy of the products are maintaining the temperature, oxygen and carbon-dioxide concentrations, and prevention of contamination. The techno-economic aspects of different cultivation systems are discussed elsewhere (see Chapter 18).

10.3.1 Finding desirable strains

Cyanobacteria inhabit many terrestrial and aquatic environments, ranging from the tropics to the polar regions. Cyanobacteria also constitute the dominant microbial flora of many extreme habitats, such as hot deserts, high-temperature water bodies, cold lakes, soda lakes, and environments with high-level UV radiation or salinity (Whitton and Potts, 2000). Understanding, managing, and taking advantage of the biology of desired cyanobacterial strains is fundamental to the success of any biofuel production program. During large-scale culturing in natural or artificial systems, the cyanobacterium should be able to tolerate variations in temperature, light, and salinity, as well as resisting pathogen attack. Strains optimized for cultivation in particular geographical regions and seasons are needed to ensure their widespread use. Thus a survey of novel and unique habitats for strains that are versatile, robust, and less susceptible to heterotrophic microbial contamination during mass cultivation is important.

Native strains are unlikely to have all of the characteristics needed for industrial production. Therefore, we need to modify these organisms through genetic engineering to provide adequate yield and quality of biomass (Georgianna and Mayfield, 2012). Breeding programs to improve phenotypes (which are not known for cyanobacteria) and genetic engineering of cyanobacteria (to increase their photosynthetic efficiency) and their metabolic pathways, could play important roles in the improvement of growth and biomass accumulation. Although there is no evidence regarding comparative growth performance of mutants (with desired traits) and their wild types, enhancing the genetic capabilities of an organism for biofuel production certainly has potential. Recently, our understanding of cyanobacterial genomes has improved rapidly, especially with regard to the genes involved in metabolic responses, which could assist greatly in the metabolic enhancement of cyanobacteria for biofuel production (Quintana *et al.*, 2011). Figure 10.2 shows steps that are crucial to obtaining economically feasible biofuel production.

10.3.2 Energy source and utilization efficiency

Fossil fuels are used in the entire production process, from culturing, nutrient requirement, harvesting, transportation, to processing. Hence an ideal cost-competitive cyanobacterial biofuel production system should be less energy intensive, with a positive energy balance, and run on renewable energy sources rather than on fossil energy. Amongst the various renewable energy sources available, solar energy is perhaps the most attractive because of its plentiful availability. If the average incident solar energy (at 170 W/m^2) could be captured with 100% efficiency, only 88,201 km^2 (0.017% of the surface of the Earth) would be required to supply total current global energy demand. However, most natural plant ecosystems have a solar-energy-to-biomass conversion efficiency of ~1% (Posten and Schaub, 2009). Since green plants only use part of the solar spectrum, namely the photosynthetic active radiation (PAR; 350–700 nm), a large area would be required to satisfy global energy demand, depending on the light-energy–biomass conversion efficiency, day length (sunshine hours), temperature, and other factors (see Stephens *et al.*, 2010 for details).

For conventional open-pond systems, the light-to-biomass conversion efficiency of microalgae is 1–4%, while the number is 8–10% for closed photobioreactors (Stephens *et al.*, 2010 and references therein). Genetic engineering of cyanobacteria to enhance their light-capturing capacity could increase these efficiency numbers (Stephens *et al.*, 2010; Quintana *et al.*, 2011 and references therein). Moreover, the use of closed photobioreactors for low-grade single products (e.g., biofuels) will be too costly, because they require a large input of fossil energy. Non-arable wastelands located in tropical areas and receiving sufficient sunshine could

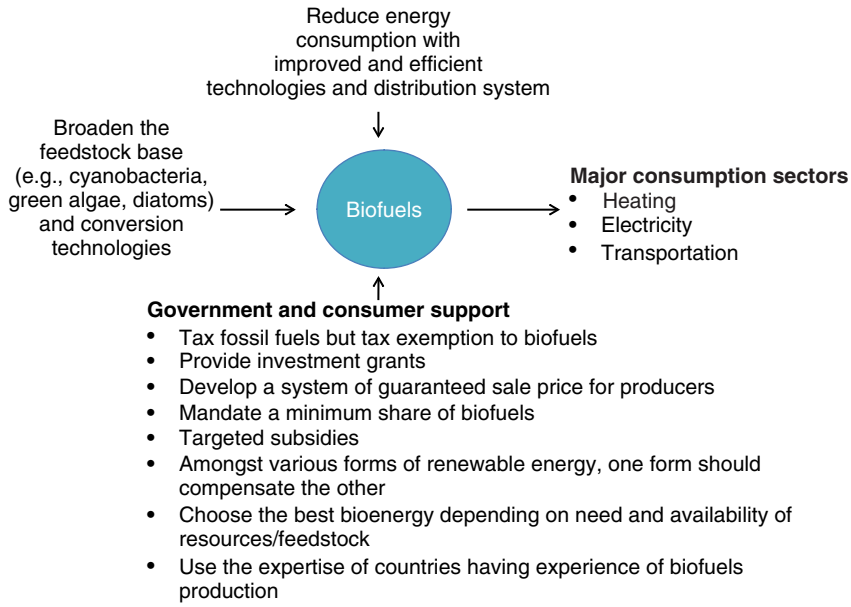
Reduce energy consumption with improved and efficient technologies and distribution system
Broaden the feedstock base (e.g., cyanobacteria, green algae, diatoms) and conversion technologies
Biofuels
Major consumption sectors
• Heating
• Electricity
• Transportation
Government and consumer support
• Tax fossil fuels but tax exemption to biofuels
• Provide investment grants
• Develop a system of guaranteed sale price for producers
• Mandate a minimum share of biofuels
• Targeted subsidies
• Amongst various forms of renewable energy, one form should compensate the other
• Choose the best bioenergy depending on need and availability of resources/feedstock
• Use the expertise of countries having experience of biofuels production

Figure 10.2 Steps important for economic production of biofuels.

be the ideal place for outdoor closed photobioreactors. Furthermore, other renewable sources, such as solar and wind energy, could be utilized to satisfy the energy demand of such photobioreactors.

10.3.3 Water requirement

The use of fresh water for cyanobacterial biofuel production is another constraint. Benemann *et al.* (1982) proposed an equation to calculate the water demand of a process:

$$Q_{in} = EA/(1 - BDR)$$

The blow-down rate (BDR) of a system is the ratio of water output (Q_{out}) to water input, (Q_{in}), with values for irrigation and salty waters of 0.1 and 0.3, respectively. Then, in a pond with a recycling facility, water is mainly lost through evaporation (E, evaporation rate), which is directly proportional to the area (A) of the pond. In recent times, use of brackish and salt water has been advocated for biofuel production. Many cyanobacterial species can grow at a wide range of salinities and can therefore be exploited for biofuel production. According to a claim by Algenol Inc, cyanobacteria can convert 2 gal of seawater into 1 gal of freshwater by evaporation and condensation of the produced ethanol (Algenol Inc, 2010). However, salt water is corrosive and therefore special pipes and pumps are required.

10.3.4 Nutrient requirement and utilization

Use of the photoautotrophic approach eliminates the need for costly organic carbon sources. Inorganic carbon from polluting industries could be used as a carbon source but this would require cultivation of the cyanobacteria in the immediate vicinity of such industries (e.g., power plants and cement factories). Unlike the production of fine chemicals, biofuel production requires only low-purity carbon dioxide (Gonzalez-Lopez *et al.*, 2009). SEAMBIOTIC was the first company to use flue gases from coal-fired power stations as a source of carbon dioxide for microalgae cultivation (Shen Goh and Lee, 2010). Similarly, many species of cyanobacteria use abundant atmospheric molecular nitrogen. Nevertheless, cyanobacteria still require phosphorus, potassium, and other micronutrients, which are expensive and limited in supply.

On the one hand, these costs can be managed by locating algal ponds or bioreactors near nutrient-rich wastewater streams (Kumar *et al*., 2010; Park, Craggs, and Shilton, 2011), or by using feed sources such as anaerobic digester waste effluents (Georgianna and Mayfield, 2012). On the other hand, some strains might have specific nutritional requirements and therefore the optimization of growth media is essential for the production of desired products (Stephens *et al*., 2010).

Recycling of water and nutrients will have positive impacts on the economics of biofuel production. After fermentative digestion, the water and nutrient-rich residue can be recycled back into the bioreactors or ponds. Moreover, many cyanobacteria excrete compounds into their environment that could inhibit their own growth (autoinhibition) or that of other organisms (heteroinhibition). Autoinhibiting compounds need to be removed from the recycled water stream, while heteroinhibiting compounds could potentially be exploited to prevent contamination. To overcome autoinhibition, strategies such as nutrient replacement, periodic ultrafiltration of the media, and adequate dilution rates in continuous cultivation can be applied (Stephens *et al*., 2010 and references therein).

10.3.5 Protection from contamination

Protection of cultures in outdoor raceway ponds and closed bioreactors is essential because it could result in major losses of productivity. However, little is known about the pathogens, grazing pests, and other organisms that could outcompete the target strain. Long-term pilot studies in various environments are needed. Compared to open systems, contamination can be better managed in closed photobioreactors. However, both open and closed systems are susceptible to contamination during long periods of continuous cultivation (Stephens *et al*., 2010). Modification of cultivation regimes, such as use of increased inoculum sizes (in closed systems) and decreased retention times in open ponds, could minimize contamination (Huntley and Redalje, 2006). Moreover, shifts in single environmental parameters (e.g., pH, temperature, solar irradiance, or osmolality) can act as a selection pressure, favoring the dominance of the target strain over competing organisms (Shapiro, 1973; Vincent and Silvester, 1979a, b). A number of cyanoviruses and grazers attack cyanobacteria. Because of their specific requirements, thermophilic and halophilic cyanobacteria might be the desired candidates for biofuel production. Moreover, it is possible to identify or breed strains that are resistant to the antibiotics used to control unwanted species.

10.3.6 Harvesting, fermentation and recovery of biofuels

Prior to converting cyanobacterial biomass to liquid transportation fuels, pre-processing steps such as harvesting and dewatering are required. Especially in raceway ponds, where the cultures of cyanobacteria are at low densities, it is necessary to concentrate the cultures (up to 30-fold). These steps are energy-intensive and therefore costly. The high-energy input for harvesting biomass has made current biofuel production uneconomic. Harvesting of cyanobacterial biomass in commercial production plants is generally done by centrifugation, which represents more than 30% of the total production costs in the case of algal production in open ponds. This needs to be minimized (Wijffels and Barbosa, 2010). Evaluation of several harvesting methods showed that flocculation combined with flotation or sedimentation and subsequent further dewatering by centrifugation or filtration is the most promising cost- and energy-efficient alternative. Flocculation can also be induced by changing the culture conditions by applying extreme pH, depleting nutrients, or changing the temperature or the level of dissolved oxygen.

Detail of various harvesting processes and their suitability is discussed in Chapter 18. Salim *et al*. (2011) used microalgae to flocculate cultures (bioflocculation). Bioflocculation is a cost- and energy-efficient approach, which does not involve the use of any chemicals. However, the applicability of these techniques at large scales needs to be tested.

After harvesting, the desired products are extracted from the biomass using either mechanical or enzymatic procedures. Major components that can be extracted from cyanobacterial biomass are carbohydrates (fuel precursors) and proteins (animal/fish feeds). It is critically important to identify the separation techniques that consume the least energy; the high energy demands associated with handling and drying of algal biomass

and separating the desired products is still a huge challenge.

Extracts from the biomass can be fermented to ethanol using a suitable yeast strain. The fermented broth is drained from the tank and pumped to a holding tank to be fed to a distillation unit for separation and purification of the alcohol. Most open ponds use a semi-continuous harvest strategy that removes roughly half of the cyanobacteria. A continuously operating system is better than a batch process and results in enhanced volumetric productivity, smaller bioreactor volumes, and lower investment and operational costs (Brethauer and Wyman, 2010). Moreover, in continuous systems cells can be immobilized by adsorption to solid surfaces, either entrapped within a porous matrix (e.g., calcium alginate, k-carrageenan, polyvinyl alcohol, agar, gelatin, chitosan, or polyacrilamide), mechanically contained behind a barrier (e.g., microporous membrane filters or microcapsules), or self-aggregated by flocculation. The immobilization retains high cell densities inside the bioreactors and is economically a decisive factor (see Kourkoutas *et al.*, 2004; Bányik *et al.*, 2005; Verbelen *et al.*, 2006 for details)

Designing an optimum cultivation system involves understanding the biology of the cyanobacterial strain used and its integration with the most suitable downstream processing options. Choices made for the cultivation system are key to the affordability, scalability, and sustainability of cyanobacterial biofuel systems. Focusing on biofuels as the end product poses challenges, due to the high volume and low value associated with bulk commodities such as gasoline and diesel fuels. Transport and storage of biofuels are other factors not discussed here but have an important bearing on the overall economics of cyanobacterial biofuels. Table 10.2 lists the techno-economic challenges involved in commercial-scale biofuel production.

10.4 Cyanobacteria as hydrogen energy source

Hydrogen energy is an attractive alternative energy source. It is an ecofriendly, efficient, renewable, carbon-neutral and commercially viable energy medium (Dutta *et al.*, 2005). Hydrogen liberates large amounts of energy per unit weight in combustion, does not evolve carbon dioxide, and can be converted directly into electricity by a fuel cell. Only microorganisms are capable of producing hydrogen. Microbial hydrogen production can either be mediated by light (e.g., photosynthetic bacteria including cyanobacteria; photo-hydrogen production), or be derived from fermentation of organic sources in the dark (e.g., using members of Enterobacteriaceae). In dark fermentation, the reaction rate is high but the yield is only 10–20% of the hydrogen stored in the substrate due to incomplete decomposition. Moreover, hydrogen yield varies with the type of substrate used. The major problems associated with this process are contamination by hydrogen-consuming microbes such as the methanogenic and acetogenic bacteria that use hydrogen to produce methane and acetate, respectively. The sensitivity of hydrogenase to oxygen makes hydrogen production difficult, while procedures to remove oxygen from cultures are expensive.

The reported cost of photobiologically produced hydrogen is much less ($\$25/m^3$) than that of photovoltaic splitting of water ($\$170/m^3$; Block and Melody, 1992). However, this study is very old, and newer comparative values are required. Cyanobacteria produce hydrogen through three main routes:

- mediated by bidirectional hydrogenase (Ananyev, Carrieri and Dismukes, 2008)
- mediated by nitrogenase (Lindberg, Lindblad and Cournac, 2004)
- through the introduction of a hydrogenase from another organism (Tamagnini *et al.*, 2007; Angermayr *et al.*, 2009).

However, the low rate of hydrogen production, possibly due to the complicated reaction system needed to overcome the large free energy of +237 kJ/mol H_2, the limited duration of hydrogen production, and the difficulties of collecting the gas are major drawbacks that affect commercialization of biohydrogen (Miyaki, 1998; Chen *et al.*, 2008).

Nevertheless, cyanobacteria-mediated hydrogen production has many advantages over other biological and non-biological processes (Benemann and Weissman, 1976; Benemann *et al.*, 1977; Karube *et al.*, 1986; Madamwar, Garg, and Shah, 2000; Dutta *et al.*, 2005). For instance, Mitsui (1980) reported that a marine

Table 10.2 Major techno-economic challenges to commercial scale cyanobacterial biofuels production.

	Process steps	Research and development challenges
Upstream processes	Algal biology	• Sample strains from a wide variety of environments • Develop small-scale, high-throughput screening technologies • Develop open-access database and collections of existing strains with detailed characterization • Investigate genetics and biochemical pathways for production of fuel precursors • Improve strains for desired criteria by gene manipulation techniques or breeding
	Cultivation	• Investigate multiple approaches (i.e., open, closed, hybrid, and coastal/offshore systems; phototrophic, heterotrophic, and mixotrophic growth) • Achieve robust and stable cultures at a commercial scale • Optimize system for productivity of fuel precursors • Sustainably and cost-effectively manage the use of land, water, and nutrients • Identify and address environmental risks and impacts
Downstream processes	Harvesting and dewatering	• Investigate multiple harvesting approaches (e.g., sedimentation, flocculation, dissolved air flotation, filtration, centrifugation, etc.) • Minimize process energy intensity • Lower capital and operating costs • Assess each technology option in terms of overall system compatibility and sustainability
	Extraction, fermentation, and fractionation	• Investigate multiple approaches (e.g., sonication, microwave, solvent systems, supercritical fluid, subcritical water, selective extraction, and secretion) • Achieve high yield of desired intermediates; preserve co-products • Minimize process energy intensity • Investigate recycling mechanisms to minimize waste • Address scaling challenges, such as operational temperature, pressure, carrying capacity, side reactions, and separations
	Fuel conversion	• Investigate multiple approaches to liquid transportation fuels (e.g., direct fuel production, thermochemical/catalytic conversion, biochemical conversion, and anaerobic digestion) • Improve catalyst specificity, activity, and durability • Reduce contaminants and reaction inhibitors • Minimize process energy intensity and emissions over the life cycle • Achieve high conversion rates under scale-up conditions
	Co-products	• Identify and evaluate the co-production of value-added chemicals • Optimize co-product extraction and recovery • Conduct market analyses, including quality and safety trials to meet applicable standards
	Distribution and utilization	• Characterize algal biomass, intermediates, biofuel, and bioproducts under different storage and transport scenarios for contamination, weather impacts, stability, and end-product variability • Optimize distribution for energy and costs in the context of facility siting • Comply with all regulatory and customer requirements for utilization (e.g., engine performance and material compatibility)
	Resources and siting	• Assess and characterize land, climate, water, energy, and nutrient resource requirements for siting of cyanobacterial (heterotrophic and photoautotrophic) production systems • Integrate with wastewater treatment and/or CO_2 emitter industries • Address salt balance, energy balance, water and nutrient reuse, and thermal management

Adapted from U.S. DOE, 2010.

cyanobacterium *Oscillatoria* grown in a chamber 1.86 m^2 × 0.91 m deep produced enough hydrogen to generate 1000 kW electricity per month.

There are several extrinsic (light, temperature, salinity, micronutrient levels, carbon and nitrogen sources, and oxygen, sulfur, and methane levels) and intrinsic factors (genetic components and sensitive proteins in cyanobacteria) affecting cyanobacterial hydrogen production (Dutta *et al.*, 2005; Angermayr *et al.*, 2009). Antal and Lindblad (2005) reported increased production of hydrogen by the unicellular cyanobacterium *Gloeocapsa alpicola* under sulfur-starvation. Likewise, *Spirulina platensis* and *Anabaena cylindrica* produce excess hydrogen under both anaerobic dark conditions (Aoyama *et al.*, 1997) and light-limited conditions (Jeffries, Timourien and Ward, 1978). The hydrogenase-deficient cyanobacterium *Nostoc punctiforme* NHM5, when incubated under high light for a long period (until the culture was depleted of carbon dioxide), shows increased hydrogen production (Lindberg, Lindblad, and Cournac *et al.*, 2004; Dutta *et al.*, 2005).

Photo-biological hydrogen production in cyanobacteria is mainly associated with nitrogenase rather than hydrogenase. The evolution of oxygen during nitrogen fixation is a major problem associated with cyanobacterial hydrogen production. Prabaharan and Subramanian (1996) manipulated the growth conditions of *Phormidium valderianum* BDU 20041 (a marine filamentous cyanobacterium) to overcome the problem of oxygen evolution. Under white light (of intensity 5.5 µmol photon/m^2/s, 18:6 h dark/light cycle, temperature 27°C; pH 7.5; salinity 25 g/l), *Phormidium valderianum* BDU 20041 produced a maximum of 0.2 µmol H_2/h/mg dry weight in the gas phase without oxygen co-production. This was on a par with the maximum yield reported in the literature. Masukawa, Mochimaru, and Sakurai (2002) reported that knocking out the *hup*L gene in *Anabaena* sp. 2120 increased hydrogen production 4–7 times over the levels seen with the wild type. This also increased energy conversion efficiency to 0.59% of total solar radiation. However, hydrogen production lasted only 30–40 h. The targeted energy conversion efficiency was 1% of total solar radiation (Masukawa, Mochimaru, and Sakurai *et al.*, 2002). Madamwar, Garg, and Shah (2000) and Dutta *et al.* (2005) have reviewed the various aspects and strategies to improve hydrogen production from cyanobacteria.

Immobilization of cyanobacteria in various matrices (e.g., polyurethane and polyvinyl foams) could be an interesting solution to the problem of fuel, energy, and chemicals. Markov (1997) patented a method for immobilization of cyanobacteria on the outer surfaces of fibers in a photoreactor. When aerated aqueous salt solution is pumped over the cyanobacteria, hydrogen produced by them seeps inside the fibers and can be collected in a vessel. This can be heated to 80–90°C to separate the hydrogen gas.

Compared to photo-hydrogen production, much less free energy is required when cyanobacteria use an organic substrate. However, this depends on the growth phase. Fructose and sucrose are the preferred substrates for hydrogen production by cyanobacteria (Chen *et al.*, 2008). These authors also reported that, owing to a lower oxygen concentration, cells at the late exponential growth phase produce more hydrogen than during the exponential phase. Addition of fructose after a saturation level raises hydrogen production again without any delay. However, a high concentration of fructose has an inhibitory effect on hydrogen production (Chen *et al.*, 2008). The maintenance of a high hydrogen production rate over a long period of time requires the selection of strains with excellent properties for hydrogen production, genetic engineering (if required), and optimization of the growth conditions.

In general, the lipid content of cyanobacteria is too low for commercial-scale biodiesel production. However, there are a few species that could be exploited and these have been described elsewhere (see Chisti, 2007; Song, Fu, and Shi, 2008).

10.5 Concluding remarks

Cyanobacteria are bestowed with a unique combination of plant-like photosynthesis and an ability to yield high amounts of desired products under controlled conditions. There have been years of research into achieving a cost-competitive (i.e., economically viable) and sustainable cyanobacterial biofuel system. Careful planning and investments in nascent but promising technologies are still required. It may not be possible to achieve these goals via one technology pathway. Therefore we need to include new and

innovative technologies in an integrated process, for example by integrating cyanobacterial biofuel production technologies with wastewater treatment systems. Wastewater essentially contains nitrogen, phosphorous, and potassium in high concentrations, and these could act as a mineral supplement for cyanobacterial biomass production. By combining metabolic engineering with advances in photobioreactor technologies the production of biofuels could be optimized. The information from pilot-scale production plants shows that the different by-products that could be generated in different stages of the production of biofuels are key to developing a cost-competitive cyanobacterial biofuel system. Thus a combination of systems biology, genetic engineering, and biorefinery development is a must to make biofuel economically viable. Moreover, lifecycle assessment studies of the energy and environmental impact of different production pathways will assist the development of commercially viable cyanobacterial biofuel production systems.

10.6 Declaration

The section "Cyanobacteria as hydrogen energy source" has been taken from one of our own articles (Sharma *et al*., 2011), with kind permission from Springer Science + Business Media B.V.

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Chapter 11

Cyanobacterial cellulose synthesis in the light of the photanol concept

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11.1 Introduction

The energetics of life on Earth have evolved as a cycle of redox reactions of the elements carbon, hydrogen, and oxygen: Sunlight drives the conversion of carbon dioxide and water into the biomass of oxyphototrophs and chemo-heterotrophic organisms re-oxidize the products of oxygenic photosynthesis back to carbon dioxide and water, in a process in which they transiently store reduced forms of carbon in chemo-heterotrophic biomass. However, during the billions of years of evolution, a significant amount of biomass/carbon has been removed from this cycle, to be deposited in reduced, fossilized forms (mainly gas and oil).

Since the beginning of the industrial revolution mankind has massively exploited the free energy contained within these fossil sources of energy, which has led to a notable increase in atmospheric carbon dioxide levels. This development has generated serious concern about global warming. Hence, and also for political reasons (Dale, 2012), there is a considerable incentive to develop sustainable alternatives to the exploitation of fossil fuel. By far the majority of the free energy available on Earth is ultimately derived from sunlight, and it is available in a large excess over the energy demands of mankind (Lewis and Nocera, 2006). It is therefore rational to exploit sunlight directly.

Many ways to achieve the direct use of sunlight have been exploited since the first oil crisis in the

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early 1970s. Hydroelectricity, photovoltaics, and wind energy are the most notable examples. All of these produce electricity, which is – together with liquid fuel (and heat) – the main free-energy carrier on Earth. There still is a need for the development of new methods for the sustainable production of a liquid fuel in the form of solar generated biofuel.

One may think that in the future liquid fuel will be produced by chemical devices (often referred to as "artificial leaves"), which would convert carbon dioxide into more reduced forms of carbon. However, in this review we will not discuss the solar-driven splitting of water into hydrogen and oxygen, either with chemical devices, or using living organisms. Despite significant effort, only limited progress has been made along these lines (Bensaid *et al.*, 2012), particularly with respect to the formation of covalent carbon–carbon bonds. Since the turn of the century the idea has gained momentum that natural oxygenic photosynthesis would be more suitable for sustainable biofuel production. The main driver for this idea is the autocatalytic nature of formation of the devices that produce the fuel in the first place, namely the living cells of plants, algae, or cyanobacteria.

In two forms this approach of exploiting natural biosynthesis already finds large-scale application: the conversion of crop plants (e.g., maize, or one of its subfractions such as (ligno)cellulose) into methane via the use of an anaerobic digester and of its polysaccharide fraction into solvents such as ethanol or butanol, via industrial acetone–butanol–ethanol (ABE) fermentation. Yet, in a third mode the lipid fraction of crops can be converted into biodiesel. However, all these methods suffer from the disadvantage that arable land and valuable crops – which can also be used for food or feed applications – are used to produce energy, in a world in which the future food supply is a serious concern.

Shifting to the use of algae or cyanobacteria would alleviate this latter problem, because these aquatic organisms can be grown in various forms of photobioreactor. Hence, activity aimed at the production of biodiesel through large-scale growth of various algae has gained significant momentum. But even in this approach there is an important element of inefficiency: The carbon fixed in the Calvin–Benson–Bassham cycle is first converted into all the complicated building blocks for biomass formation (amino acids, lipids, sugars, nucleotides, vitamins, etc.), after which cellular anabolism converts these "monomers" into new cells. These cells then have to be processed, in other words they are fractionated and/or fermented, after which another processing step (such as ethanol fermentation) is required to make the final product.

11.1.1 The "photanol" concept

To further increase the overall efficiency of the process of solar biofuel formation it has been proposed to combine phototrophic and fermentative metabolisms in a single organism: a kind of "photofermentative" chimera. If the activity of the added fermentative pathway can be tuned properly, one could achieve a situation in which such a photofermentative organism functioned as a photo-catalyst for the reaction: $CO_2 + H_2O \rightarrow$ "biofuel product" $+ O_2$. This proposed concept has suggested as being applicable to the synthesis of various products, ranging from lactic acid and ethanol, for which a simple fermentative metabolic pathway suffices (Hellingwerf and Teixeira de Mattos, 2009), via sugars (Ducat *et al.*, 2012), to longer chain alkanes and alcohols (Machado and Atsumi, 2012). Elsewhere, we have referred to this concept of solar biofuel production as the "photanol concept" and several initiatives have been undertaken to test its economic viability. The concept can be applied to all oxyphototrophs, including plants (in which the synthesis of poly-β-hydroxy butyrate can be considered as an example), but is most easily applied in cyanobacteria because of their much better genetic accessibility and more simple structure.

Generally, fermentative pathways function best when oxygen is absent. The photanol concept was conceived from the observation that organisms such as yeasts also use fermentative ethanol production extensively under aerobic conditions, when glucose is in excess. Nevertheless, the compatibility of a heterologously expressed metabolic pathway with the condition of high oxygen concentration in the cytoplasm is an important element in the design of a photofermentative chimera: the chance of functionally expressing the well-known clostridial butanol fermentation pathway in an organism that carries out oxygenic photosynthesis will be small. Even less likely is the

possibility of combining methanogenesis with oxygenic photosynthesis, because in this case, not only the oxygen-sensitivity of the methane-forming enzymes must be addressed, but also the incompatibility of the metabolic intermediates and the cofactors.

11.2 Maximizing photosynthetic efficiency

Light, as a source of energy in biology, is rather delicate to handle because it comes in packages (photons), many of which carry much more free energy than what is contained in, for example, a single covalent C–C bond. Hence various forms of non-productive chemistry are readily initiated by (electronically excited) molecules, the most notable of which is the formation of reactive oxygen species, such as singlet oxygen and superoxide. In agreement with this notion, many regulatory processes have been discovered in phototrophic organisms that allow for the processing of this radiation energy directly into heat if – temporarily – the forward reaction towards the productive biological process is blocked. The best known example of such processes is the non-photochemical quenching of excitons in the antenna system of many oxyphototrophs, when – due to transient over-excitation of particularly photosystem II – the primary electron acceptor of this system is already in the reduced form at the time of arrival of the next electron. But such processes have also been identified much later in the chain of events that converts the solar radiation into the chemical free energy of NADPH and ATP (i.e., the two free-energy intermediates that drive the reactions in the Calvin–Benson–Bassham cycle), for example the Mehler reaction. This latter reaction transfers excess electrons arriving from photosystem I directly to oxygen, thereby producing superoxide radicals, which are disproportionated to hydrogen peroxide and water. Additional pathways exist, mediated by Flv proteins, in which oxygen is reduced directly to water without formation of oxygen radicals (Helman *et al.*, 2003). Nevertheless, less energy-dissipating forms of regulation have also emerged, such as the state transitions, in which, through covalent modification of antenna proteins, the cross section of the two photosystems is adjusted via reversible migration of the antenna pigments to these photosystems, so that the rate of excitation of the two photosystems is in balance, under widely varying conditions of the spectral composition and intensity of the available ambient radiation.

Successful application of the photanol concept will require that the photosynthesis process in future cyanobacterial cell factories proceeds with the utmost efficiency, preferably so that all photons present in the spectral window of photo-synthetically active radiation will be used productively and not be dissipated into heat prematurely. Intrinsically, for thermodynamic reasons, the process of oxygenic photosynthesis is already so inefficient that it can never be much higher than 30%. Actual photosynthetic yields in agriculture rarely exceed 1% and the theoretical maxima for crops are 4.5% and 6% for C(3) and C(4) plants, respectively (Zhu, Long, and Ort, 2010). For cyanobacterial photosynthesis, yields up to 10% of the total radiation impinging on the Earth's surface have been reported in small lab-scale photo-bioreactors, and it is a significant challenge to design photo-bioreactor systems in which such high yields will be attainable at large scale (and at an affordable price). A significant hurdle in meeting that challenge is the dynamical fluctuations in light intensity to which cells in such large-scale reactors will be exposed. The two main factors that govern these fluctuations are:

- the limited penetration depth of the light in dense cell cultures
- the circadian and seasonal alterations in light color and intensity.

It will take intelligent reactor design to make the most of this optimization challenge.

To complicate matters even further, biological engineering is also among the tools that are available to make this optimization process a success. A very clear example of the use of this technique has already been carried beyond the stage of proof of principle: by varying the total complement of antenna pigments, the penetration depth of sunlight into a photo-bioreactor, and thereby the relative fraction of photosynthetically productive cells in it, can be increased (Ort and Melis, 2011). However, with this approach the possibilities are by no means exhausted. Obvious candidate approaches are alteration of the mutual stoichiometry and content

of the two photosystems and alteration of the color of the photosynthetic pigments (Blankenship *et al.*, 2011). Optimization of the "dark reactions of photosynthesis" (such as the *in vivo* activity of RuBisCO) are also among the possibilities, or even introduction of far-red-absorbing proton-pumping rhodopsin into the cyanobacterial membrane(s).

11.3 Downstream processing of solar biofuel products

11.3.1 Low-molecular-weight biofuel products

The "distributed" nature of sunlight has important consequences for the geometry of solar bioreactors in which cyanobacteria can be grown under ambient solar irradiation and the optimal cell densities in these bioreactors. High cell densities cause a limited penetration depth of light into the photobioreactor, whereas large volumes of cells in a photobioreactor that would not receive light will only contribute to (maintenance) energy dissipation, rather than product formation. Therefore, most large-scale facilities for growth of unicellular oxyphototrophs have an average aerial depth of between 0.1 and 0.3 m, which can be obtained by many different ways of arranging tubes, plates, or even plastic bags.

Solar biofuel production differs considerably from traditional ABE fermentation. In ABE processes, fermentative bacteria convert high concentrations of polysaccharide or sugar into solvents such as ethanol and butanol, such that these products – often facilitated by high solvent tolerance of the fermentative bacteria or yeasts – accumulate to high concentrations. Furthermore, the high biomass density causes substantial heating of the compact reactors. Recovery of the biofuel products can then be achieved through distillation, filtration and/or pervaporation techniques. The economic feasibility of this downstream processing is inversely correlated with the solvent tolerance of the fermentative organism selected. This tolerance can be increased by random screening or through rational engineering. In this respect it is relevant to note that a yeast strain was recently described that tolerates up to 17% (v/v) ethanol (Pereira *et al.*, 2012).

As of yet, the product levels achieved in applications of the photanol concept using a cyanobacterium have led to modest product accumulation levels (Dexter and Fu, 2009). Nevertheless, investigations aimed at increasing the product (ethanol) tolerance in cyanobacteria may become relevant in the future. In contrast to its conventional biofuel counterpart produced from, for example, cellulose with a chemotrophic organism, the solar biofuel product is not produced in a complex broth, but rather in a solution in which the solar biofuel product – after cell-separation – is the dominant component. Ethanol recovery after photofermentation is probably best achieved by multistep separation, or by evaporation (using solar heating) and subsequent condensation (Woods *et al.*, 2004).

One way to circumvent downstream processing problems is to synthesize a volatile or insoluble product, allowing phase separation. Various products can be used for phase separation, for instance ethylene or butylene. Proof of principle for the production of some of these products has already been given (Wang *et al.*, 2000). However, the requirements for the maintenance of a proper gas atmosphere in mass cultures of cyanobacteria for solar biofuel production – in other words a high level of carbon dioxide in order to avoid photorespiration and preventing super-saturation of oxygen – makes it difficult to recover volatile products. Nevertheless, there is hope that newly developed methods will circumvent these problems and optimize product recovery and cultivation conditions (Bentley and Melis, 2012).

Another way out of the high costs of product recovery from dilute solutions may be found in the coupling of processes, so that the dilute solar biofuel product is converted by a high-affinity biological process into a secondary product in a (photo)chemical conversion in which product separation can be based on phase separation (Bekker, M. and Hellingwerf, K.J., manuscript in preparation).

11.3.2 Macromolecular solar biofuels

A radically different approach would be to synthesize a macromolecular compound such as a polysaccharide,

a polyester, or a lipid. These macromolecules are more easy to separate and minimize the energy expenditure for downstream processing. Examples include the conversion of the Calvin–Benson–Bassham cycle intermediate(s) into poly-β-hydroxy butyrate, poly-(D,L)-lactic acid, glycogen, or long-chain triglycerides. Nevertheless, such an approach would suffer from the disadvantage that it relies on the storage capacity of the cell. Moreover, a high intracellular content of the polymer would be required in order to make its extraction economically feasible. In order to circumvent the restriction of cellular capacity, a polymer that is deposited extracellular therefore has the preference.

It has long been known that certain chemoorganotrophic bacteria, in particular members of the genera *Acetobacter/Gluconobacter*, are able to produce cellulose, which is deposited just outside the cell envelope. When grown with non-limiting amounts of glucose, *Gluconacetobacter xylinus* produces and deposits massive quantities of the insoluble ("crystalline") cellulose (Ross, Mayer, and Benziman, 1991). The genes involved in cellulose synthesis in these bacteria as well as their regulation, have been characterized (see below).

In contrast, little is known about cellulose production in cyanobacteria. Although it has been known for a long time that cyanobacteria can produce cellulose (de Winder *et al.*, 1990; Nobles, Romanovicz, and Brown, 2001), the mechanism and regulation of the process have never been investigated. The literature on cellulose production in cyanobacteria reports production of non- or semi-crystalline cellulose (de Winder *et al.*, 1990; Nobles, Romanovicz, and Brown, 2001). A reason for the semi-crystalline nature of the cellulose may be the complex mixed-polysaccharide cell-wall layers produced by cyanobacteria. This might also complicate its purification should it be used for biofuel production. Nevertheless, a genetic approach has already been implemented to produce or increase production of cellulose from cyanobacteria (Nobles and Brown, 2008; Su *et al.*, 2011). This was achieved by introducing the *bcsAB* genes of *G. xylinus* into the cell via conjugative transfer, with *E. coli* cells harboring the cargo plasmid. Cellulose was produced in these transgenic strains but only in non-crystalline form.

Although the mechanism of crystallization of cellulose remains unknown, the observations described above now allow us to formulate an alternative biosolar cell for the production of macromolecular biofuel (see Figure 11.1). By bypassing the endogenous regulation of cellulose synthesis or through regulated heterologous expression of cellulose synthesis genes, it should be possible to construct a cyanobacterial cell that converts a large portion of its fixed carbon into exocellulose. Whether or not the latter macromolecule can be used directly or whether it first must be converted into a more convenient-to-handle liquid fuel remains to be seen. One approach that may help to increase production of cellulose, albeit non-crystalline, would be to knockout the pathway of photosynthetically fixed carbon to glycogen, the main carbon and energy storage compound of cyanobacteria (Quintana *et al.*, 2011). The substrates for glycogen and cellulose synthesis are similar: ADP-glucose and UDP-glucose, respectively. In this respect introduction or over-expression of UDPG-pyrophosphorylase might also be a successful approach.

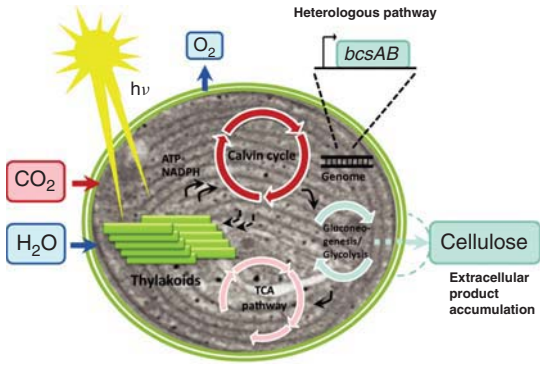

Figure 11.1 The cellulose synthesizing biosolar cell. See plate section for color version.

11.4 Cellulose synthesis

11.4.1 Cellulose structure and physiological function

Cellulose is one of the most abundant polymers on earth. It is an important component of cell walls of plants and algae, but it is also produced by a variety of bacteria and by some fungi and animals (Brown, 2004). Cellulose is a complex compound with a wide variety of structures, such as wood, paper, and cotton. In contrast to this complexity the basic structure of a

cellulose fibril is simple: it consists of only one ingredient, namely D-glucose. The D-glucose monomers are linked at a 180° angle in β-1,4 glucosidic bonds to form long un-branched polymeric chains. The special geometry of this unbranched covalent arrangement gives rise to extended fibril structures, because all of the available hydroxyl groups upon adjacently aligned β-1,4-glucan chains participate in inter- and intra-chain hydrogen bonding. In this way, aggregates of many chains form insoluble layered sheets reinforced by the dispersion forces between their stacked heterocyclic rings (Ross, Mayer, and Benziman, 1991). Nearly all cellulose isolated from nature has a crystalline structure in which the poly-β-1,4-glucan chains are aligned in a parallel manner; this is known as cellulose type I. This crystalline cellulose is metastable and can be transformed to cellulose type II, for instance by acid treatment. Cellulose II is rare in nature and has an antiparallel arrangement. For cellulose microfibrils to be assembled in such a metastable way the individual glucan chains must be held in close proximity by intermolecular hydrogen bonds and van der Waals forces (Ross, Mayer, and Benziman 1991). Therefore these chains must be synthesized in close proximity to one another. In 1964 the use of the freeze-fracture technique made it possible to investigate the surface of cellulose synthesizing cells. As a result, highly ordered membrane-bound structures were observed and the "ordered granule hypothesis" for the assembly and orientation of cellulose microfibrils was proposed (Preston, 1964; Tsekos, 1999). This vision was later confirmed with freeze-fracture experiments on the alga *Oocystis*, in which the so-called linear terminal complexes (TC) – multi-subunit arrays at the end of microfibrils – were observed (Brown and Montezinos, 1976; Delmer, 1999). Differences in the arrangements of terminal complexes lead to different shapes and sizes of the final crystalline product.

To obtain more knowledge about cellulose production and fibril assembly, attempts were made to produce cellulose *in vitro* using membrane preparations. This approach was frustrated by the fact that only very low amounts of cellulose could be produced from bacterial preparations. In cellulose preparations derived from higher plants little or no cellulose synthase activity was observed against the high background of callose (β-1,3-glucan) synthase activity (Delmer, 1999). Callose synthesis is induced as a result of plant wounding. However, *in vitro* production of cellulose is possible: in bacterial preparations by the addition of the regulatory compound c-di-GMP or its pre-cursor GTP (Ross *et al.*, 1987) and in plants by using *in vitro* cultures of fertilized cotton ovules (Beasley, 1971) instead of plant-cell preparations.

While the function of cellulose in plants as part of their rigid cell wall is obvious, its role in bacteria, and particularly in cyanobacteria is less clear. Bacterial extracellular cellulose may play a role as a protective shield against environmental stresses and external (mechanical) forcing, or it may facilitate the adhesion of the bacterial cell in case of infection or for symbiotic interactions, as is the case with *Agrobacterium* sp. and *Rhizobium* sp. (Ross, Mayer, and Benziman, 1991). In the case of *Gluconacetobacter xylinus* it has been suggested that the massive amounts of cellulose that this organism produces when growing in a static culture, and which forms a floating pellicle, helps this obligate aerobic bacterium to move to the surface in order to obtain access to oxygen (Ross, Mayer, and Benziman, 1991). One interesting hypothesis is that the cellulose sheath might protect the cell against UV irradiation (Ross, Mayer, and Benziman, 1991). This, in particular, is interesting for cyanobacteria because these phototrophic organisms are often exposed to high levels of UV light.

The presence of cellulose in cyanobacteria has gone unnoticed for a long time. One of the first detailed reports was the discovery of cellulose in a new cyanobacterial isolate, *Crinalium epipsammum*, which was shown to accumulate cellulose to 21.6% of its dry weight (de Winder *et al.*, 1990), but before that, the presence of extracellular cellulose had been suggested in heterocystous cyanobacteria, based on light and electron microscopy (Tuffery, 1969). One report suggested that cellulose is an integral component of the heterocyst cell wall and essential for protecting nitrogen fixation in these specialized cells from oxygen inactivation (Granhall, 1976), but subsequent detailed studies of the heterocyst envelope did not confirm the presence of cellulose. However, genes encoding cellulose synthase appear to be widespread among cyanobacteria and representatives of four out of the five sections of cyanobacteria were shown to produce cellulose (Nobles, Romanovicz, and Brown, 2001). *C. epipsammum* is a filamentous, non-heterocystous, cyanobacterium from section III. It was

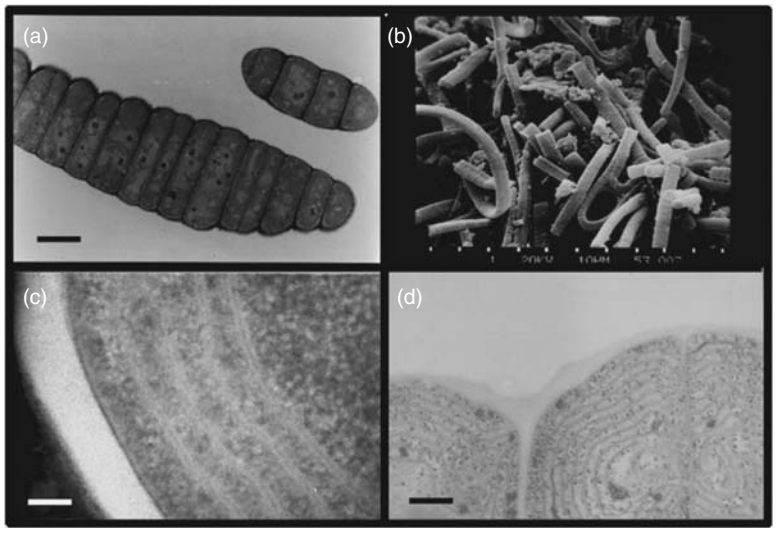

Figure 11.2 (a) Transmission electron microscopy image of *Crinalium epipsammum*. The thick cell wall with some sheath material is notable. The thylakoids and carboxysomes (polyhedral bodies) are clearly visible. (b) Scanning electron microscopy image showing the flat morphology of the trichomes of *Crinalium epipsammun*. (c) Detail of the extraordinarily thick cell wall of *Crinalium epipsammum*, which is more similar to those of Gram-positive microorganisms (cyanobacteria have a Gram-negative cell envelope). Some sheath material is visible on the outer layer of the cell wall. (d) Transmission electron microscopic image stained for polyglucose. The glycogen granules are visible between the thylakoids and the cell wall is also lightly stained, which might hint at the presence of cellulose. The scale bars in a, c, and d are 5, 0.5, and 0.5 µm, respectively.

isolated from algal crusts that grow on the sand surface of blow-outs in the dunes near The Hague, The Netherlands (Figure 11.2). These algal crusts are characterized by harsh conditions, especially drought, large temperature fluctuations, and low nutrient availability. One of the noticeable characteristics of *C. epipsammum* is its desiccation resistance. This organism is immotile and incapable of fixing nitrogen. *C. epipsammum* does not contain phycoerythrin and its phycobiliprotein content is low. Also, the DNA of this organism has an exceptionally low GC content and these characteristics may reflect its low-nitrogen habitat and its incapability of fixing nitrogen.

The cellulose detected in *C. epipsammum* might be present in non-crystalline form (de Winder *et al.*, 1990); the presence of cellulose with low crystallization has also been reported for other cyanobacterial strains (Nobles, Romanovicz, and Brown, 2001). In native form neither cellulose I nor II was detected in *C. epipsammum* but in its extracted form cellulose II was found, probably as the result of the alkali treatment. The location of the cellulose in *C. epipsammum* is uncertain. While in other bacteria the cellulose is exuded outside the cell, this was apparently not the case in *C. epipsammum*. It was hypothesized that the cellulose might be an integral part of the cell envelope. The cell envelope and the genes involved in synthesizing it in cyanobacteria reflect the environmental conditions under which the organism thrives (Yang *et al.*, 2007). *C. epipsammum* has an unusual morphology; instead of cells that are round in cross section, *C. epipsammum* is characterized by a flat, oval form. Considering the turgor, this would require special adaptation of the cell wall, which is unusually thick in *C. epipsammum*. Hence the function of cellulose in *C. epipsammum* could be the formation of the rigid cell wall, although it is unknown how this could be achieved. Moreover, it is also unknown what the function of this flat, band-like trichome could be. Another possible function of cellulose related to the habitat of *C. epipsammum* could be its water-retaining capacity; this might help the organism to withstand periods of drought (Cannon and Anderson, 1991).

In the thermophilic unicellular cyanobacterium *Thermosynechococcus vulcanus*, cellulose accumulates particularly under illuminated, low-temperature conditions and results in cell aggregation (Kawano *et al*., 2011). It has been suggested that this is an acclimation to light stress by self-shading of the cells in the aggregates (Hirano *et al*., 1997). The disruption of a putative cellulose synthase gene in *T. vulcanus* prevents the accumulation of cellulose as well as the aggregation of cells. Cellulose has also been suggested as being important as a biofilm matrix (Branda *et al*., 2005).

In the Precambrian, the oxygen-free atmosphere was devoid of ozone and UV could therefore reach the Earth's surface. Cellulose might have evolved in Precambrian cyanobacteria in order to protect them from UV light. No compelling evidence for any of these possibilities is available.

The cellulose synthase gene of *Acetobacter xylinum* has been genetically transformed into the filamentous heterocystous cyanobacterium *Anabaena* PCC7120, leading to expression and production of extracellular cellulose (Su *et al*., 2011). The transgenic *Anabaena* showed increased photosynthetic efficiency and growth rate, but it is unclear whether this was caused by the production or presence of cellulose.

11.4.2 The cellulose synthesis genes

The protein responsible for cellulose synthesis was first discovered in bacteria in 1989 (Lin and Brown, 1989) and was shown to bind UDP-glucose (Lin *et al*., 1990). The operon including the gene encoding cellulose synthase was identified through a complementation study of a cellulose-deficient mutant of *Gluconacetobacter xylinus* (Wong *et al*., 1990). Subsequently, cellulose synthase operons were identified in other bacterial species such as *Agrobacterium tumefaciens* and *Rhizobium leguminosarum* bv *trifolii* (Matthysse, 1995; Ausmees *et al*., 1999). All cellulose producing organisms require a cellulose synthase, which forms the β-1,4-glucan chain from UDP-glucose (bcsA in bacteria and cesA in eukarya). Extracellular cellulases are thought to break the glucan chains to avoid excessive mechanical stress during the crystallization process (Delmer, 1999). In bacteria, the gene for cellulase, often named *bcsZ*, is located within, or adjacent to, the cellulose synthase operon (Standal *et al*., 1994; Matthysse, 1995; Ausmees *et al*., 1999). In plants, mutations in cellulase genes are known as korrigan, and can cause dwarfism, radial swelling of root tips, and collapse of xylem vessels (Taylor, 2008). Besides *bcsA* and *bcsZ*, several other genes are present within the cellulose synthase operon in bacteria, namely *bcsB*, *bcsC*, *bcsD* in *G. xylinus* (Saxena *et al*., 1994) and *celD and celE* in *A. tumefaciens* and *R. leguminosarum* (Matthysse, 1995; Ausmees *et al*., 1999).

BcsB used to be considered the regulatory subunit of cellulose synthase because it had been reported that it binds the regulatory molecule c-di-GMP (Saxena *et al*., 1994; Delmer, 1999). However, this observation was later questioned since the 90 kD polypeptide that co-purifies with cellulose synthase does not bind c-di-GMP (Mayer *et al*., 1991). Amikam and Galperin (2006) identified a novel c-di-GMP binding domain in bacteria: PilZ. This domain was also identified near the C-terminus of the bcsA protein of *G. xylinus*. This strengthens the idea that bcsB is not the regulatory subunit of cellulose synthase. Hitherto, no alternative function for bcsB, which is required for cellulose synthesis in *G. xylinus*, has been proposed.

BcsC, which is required for *in vivo* but not *in vitro* cellulose synthesis, is present in enterobacteria, pseudomonads and in *G. xylinus* (Römling, 2002). BcsC is related to bacterial proteins involved in pore formation such as virB from *A. tumefaciens*, tra2 from *Escherichia coli*, and ptl from *Bordetella pertussis* (Saxena *et al*., 1994). Considering this, one could think that bcsC facilitates extrusion of the glucan chain into the extracellular environment.

For bcsD, which is required *in vivo*, but not *in vitro* for cellulose biosynthesis in *G. xylinus* (Saxena *et al*., 1994), and celDE, no further description has been published and no functional homologues have been identified.

11.4.3 Molecular biology, biochemistry, and phylogenetic analysis

The most extensively researched and best characterized protein involved in cellulose biosynthesis is cellulose synthase (Ces), which is a membrane-bound protein

located in the plasma membrane. The N-terminus of the plant- and some of the green-algal Ces proteins contain a RING-type zinc finger that is not present in Ces proteins from other organisms. This domain is followed by two trans-membrane domains and the central cytosolic domain, then another six trans-membrane domains and the C-terminus, at the position where in bacterial Ces proteins a PilZ domain is located (Figure 11.3). Among different organisms the cellulose synthases show low homology, with exception of the central cytosolic "catalytic" domain (Taylor, 2008). This domain contains the catalytic site present in all processive glycosyl-transferases known as the D,D,D,QXXRW motif (Saxena and Brown, 1995). This domain can be split into four sub-domains, U1–U4 (Figure 11.3), in which the first three subdomains each contain one aspartic acid and the last domain contains the QXXRW motif in which X can be any amino acid (Römling, 2002). These subdomains can be interspaced with different organism-specific regions (Figure 11.3). The *ces* genes from some cyanobacteria, green algae, plants, and the amoeba *Dictyostelium discoideum* all contain the CR-P (plant conserved region) between the U1 and U2 domains (Blanton *et al.*, 2000; Nobles, Romanovicz, and Brown, 2001; Roberts, Roberts, and Delmer, 2002). Between the U2 and U3 domains in plants and in some green algal *ces* genes, a class-specific region (CSR, previously known

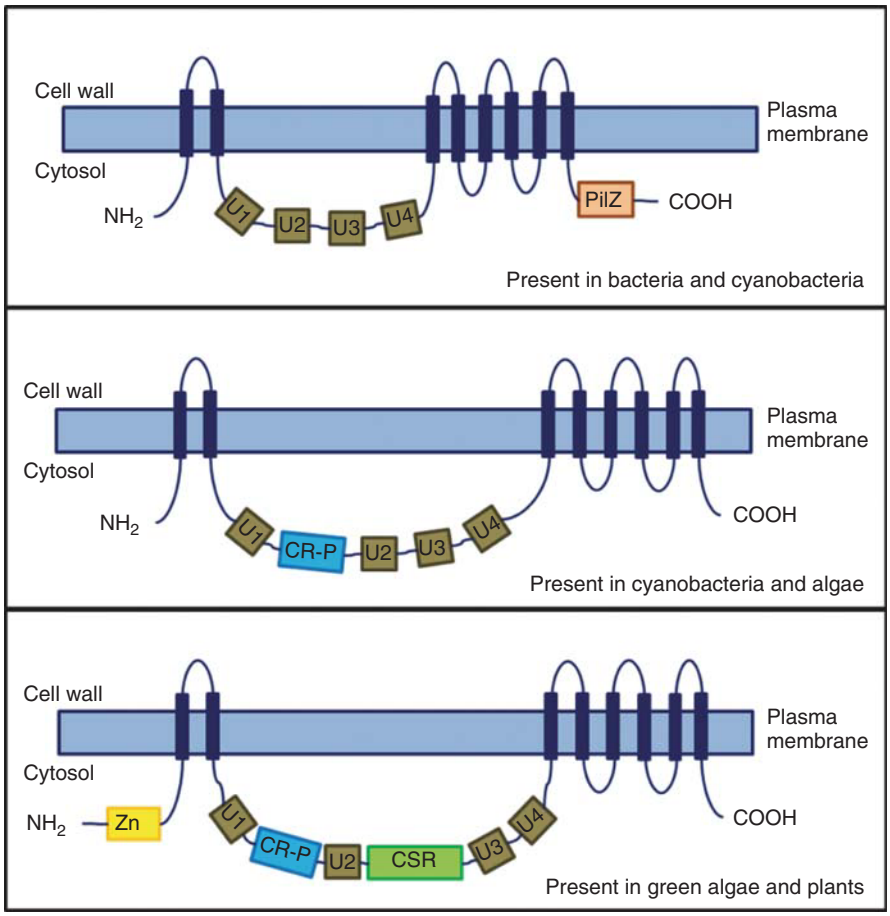

Figure 11.3 Membrane topology of cellulose synthase proteins. Blue bars indicate trans-membrane segments. PilZ, PilZ domain; Zn, RING-type zinc finger; U1–U4, catalytic domain; CR-P, plant specific region; CSR, class specific region. (Adapted from Taylor (2008). Reproduced with permission of John Wiley & Sons.) See plate section for color version.

as hypervariable region) is present. These eukaryotic genes also contain several introns (Roberts, Roberts, and Delmer, 2002; Yin, Huang, and Xu, 2009). The presence of different types of cellulose synthases in cyanobacteria and algae and the appearance of the interspacing regions within the catalytic domain provide an insight into the evolution of the cellulose synthases.

In 2001 Nobles, Romanovicz, and Brown demonstrated that cellulose biosynthesis is a common feature in cyanobacteria and, most importantly, phylogenetic analyses suggest that the cellulose synthase genes from plants are derived from cyanobacteria (Yin *et al.*, 2010; Nobles, Romanovicz, and Brown, 2001; Nobles and Brown, 2004). In cyanobacteria two types of cellulose synthases have been identified (Nobles and Brown, 2004). For example, the cellulose synthase of *Thermosynechococcus elongatus* (accession# NP_682585.1) has the same domain topology as other bacterial bcsA proteins, while the *ces* gene of the diazotrophic cyanobacterium *Nostoc* sp. PCC 7120 (accession# NP_487797.1) has lost its PilZ domain and gained an insert between the U1 and U2 domains, giving it the same domain topology as many algal Ces proteins. In phylogenetic studies comparing the cellulose synthases of all known cellulose-synthesizing classes of organisms it became evident that the eukaryotic cellulose synthases, with the exception of the Ces proteins from *C. intestinales* and *A. fumigatus*, have a unique common ancestor in the cyanobacterial *ces* gene that contains the CR-P region (Nobles and Brown 2004).

The appearance of the CSR domain within the catalytic domain and the N-terminal RING-type zinc finger within the *ces* gene seem to be related to the configuration of the active cellulose synthase complexes on the plasma membrane, known as terminal complexes (TCs). By freeze-fracturing of the plasma membrane of cellulose producing cells the TCs can be seen as multi-subunit arrays at the ends of the microfibrils (Brown and Montezinos, 1976). In bacteria the TCs are arranged in a straight line along the plasma membrane (Ross, Mayer, and Benziman, 1991; Saxena *et al.*, 1994) but in higher plants they form hexagonal arrays known as rosettes (Mueller and Brown, 1980). The largest variety in TCs is found in green algae, which exhibit different sizes of linear terminal complexes, hexagonal rosettes, and even octagonal rosettes in *Coleochaete scutata* (Figure 11.4; Okuda and Brown, 1992; Tsekos, 1999). In eukaryotes the cellulose synthase enzyme complexes are formed in the endoplasmatic reticulum and then transferred to the Golgi apparatus, where they form large globules or tetrads. These globules and tetrads are transported to the plasma membrane where they unfold and aggregate to form the active TC (Tsekos, 1999). This controlled formation of TC in eukaryotes may have enabled this high variety in TC formation in green algae, which also led to the development of the rosette. Rosettes are thought to be fully assembled before they reach the plasma membrane (Taylor, 2008). In order to form a rosette TC, two units of three different types of Ces proteins are required. These different Ces proteins most likely bind to each other through their zinc-finger domains (Kurek *et al.*, 2002). If one of these Ces proteins is missing, no rosette is formed (Taylor *et al.*, 2003).

The proteins that comprise the rosette in plants are referred to by CesA followed by a number. In *Arabidopsis*, CesA1, CesA3, and CesA6-like (CesA2,5,6, or 9) proteins form the rosette responsible for primary cell-wall formation, while CesA4, CesA7, and CesA8 form rosettes for secondary cell-wall deposition. Because of the different order of discovery of these genes in different organisms, the numbering has become confusing. The CSR in the catalytic domain appears to be specific for the different types of CesA proteins but is highly variable between different CesAs (Vergara and Carpita, 2001). It would be useful to renumber the rosette-forming CesAs based on their CSR.

11.4.4 Physiological regulation

In bacteria, cellulose synthesis is regulated by cyclic diguanylic acid (c-di-GMP). C-di-GMP was first discovered as an enhancer of cellulose synthesis in *G. xylinus* in 1987 by the group of Benziman (Ross *et al.*, 1987). Turnover of this second messenger was thought to be mediated by diguanylate cyclase (DGC), which forms c-di-GMP from two molecules of GTP, and phosphodiesterase (PDE), which linearizes c-di-GMP to 5′-pGpG. pGpG is then further degraded to 5′-GMP by non-specific cellular PDEs (Ross *et al.*, 1987; Ross, Mayer, and Benziman, 1991). In 1998, the genes that encode these enzymes in *G. xylinus* were identified (Tal *et al.*, 1998). In total three operons that mediate c-di-GMP turnover were identified and termed

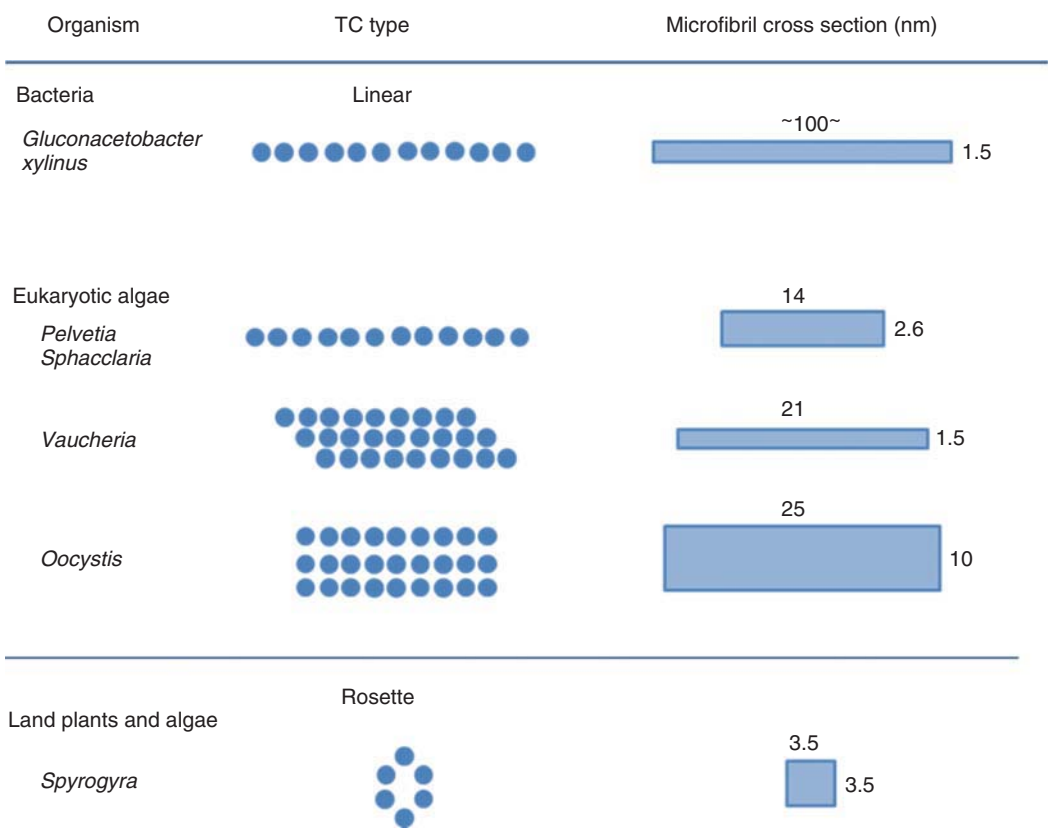

Figure 11.4 Terminal complex organization on the cell membrane in different organisms and cross sections of the resulting cellulose microfibrils. (Adapted from Tsekos (1999). Reproduced with permission of John Wiley & Sons.)

cdg1–3. Each operon encodes one PDE protein followed by one DGC protein and all turnover proteins contain both a GGDEF and an EAL domain (Tal *et al.*, 1998). These domains are named after their conserved sequence motifs and they form the catalytic site of the DGC and PDE enzymes, respectively.

The nature and the location of the c-di-GMP receptor of bacterial cellulose synthase, however, remained a point of discussion. There was some evidence that the bcsB subunit of the cellulose synthase in *G. xylinus* could bind c-di-GMP (Mayer *et al.*, 1991), but this has never been confirmed. In 2006, the first c-di-GMP binding domain was identified through a bioinformatics approach (Amikam and Galperin, 2006). This domain was named PilZ and found to be encoded within the *bcsA* subunit of the cellulose synthase in *G. xylinus*. In recent years, interest in c-di-GMP has increased greatly after it became apparent that c-di-GMP does not just regulate cellulose synthesis but that it is part of a far more ubiquitous second messenger system in bacteria (reviewed by Römling, Gomelsky, and Galperin, 2005; Hengge, 2009; Schirmer and Jenal, 2009).

The GGDEF and EAL domains are located C-terminally from, often multiple, sensory and signal transduction domains, including PAS, BLUF, haemerythrin, GAF, CHASE, and MASE. These different N-terminal domains are capable of responding to a wide range of signals: phosphorylation, protein binding, binding of gaseous molecules, or even light (Figure 11.5). PilZ domains are often associated with regulatory, catalytic, or transport domains (Amikam and Galperin, 2006) and PilZ-containing proteins function in a variety of cellular processes including the virulence of animal and plant pathogens, motility, and the synthesis of exopolysaccharides, such as cellulose or

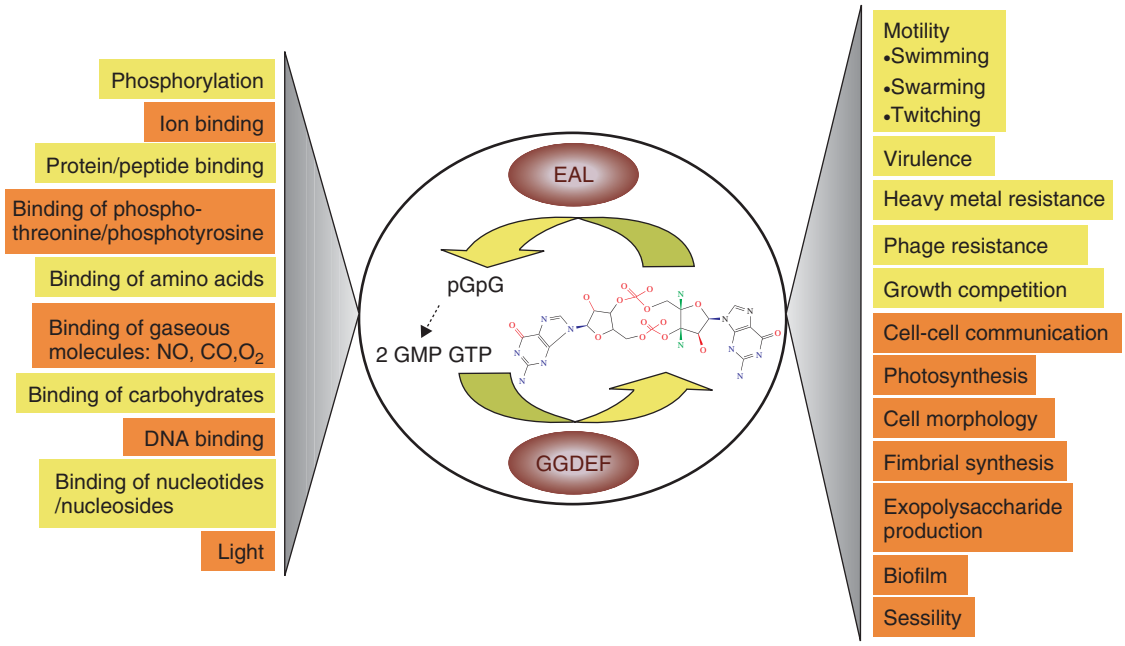

Figure 11.5 Known input signals and output of c-di-GMP metabolism. GGDEF and EAL domains conduct the turnover of c-di-GMP. pGpG is degraded to two GMP by an unknown phosphodiesterase. Various domains N-terminal of GGDEF or EAL receive and transmit the input signals (left): phosphorylation [Rec, CheY-homologous receiver domain; HisKA, histidine kinase A (phosphoacceptor) domain; HATPase, histidine kinase-like ATPases; Hpt, histidine phosphotransfer domain], ion binding (haemerythrin, HHE cation-binding domain), protein/peptide binding (TRP, tetratrico peptide repeats; CBS, domain in cystathionine beta-synthase and other proteins; CHASE, cyclase/histidine kinases-associated sensory domain), binding of phosphothreonine/phosphotyrosine (FHA, fork-head-associated domain), binding of amino acids (PBPb, bacterial periplasmic substratebinding proteins), binding of gaseous molecules [PAS/PAC, Per (periodic clock protein), Arnt (Ah receptor nuclear translocator protein), Sim (single-minded protein); haemerythrin], binding of carbohydrates (7TMR-DISMED2, 7TM receptors with diverse intracellular signalling modules), DNA binding (HTH–LUXR, helix–turn–helix, Lux regulon), binding of nucleotides/nucleosides (GAF, domain present in phytochromes and cGMPspecific phosphodiesterases; cNMP, cyclic nucleotide-monophosphate binding domain; CBS), light (BLUF, sensor of blue-light using FAD). The output behaviour by variation of c-di-GMP concentration is shown on the right. (From Römling, *et al.* (2005). Reproduced with permission of John Wiley & Sons.)

alginate (Figure 11.5; Schirmer and Jenal, 2009 and references therein). Finally, the FleQ regulator from *P. aeruginosa* (involved in transcription control) and RNA aptamers have been found to specifically bind c-di-GMP (Hickman and Harwood, 2008; Sudarsan *et al.*, 2008).

GGDEF and EAL domains are abundant in bacterial genomes, but absent from the genomes of Archaea and Eukarya (Galperin, 2004), and the same is true for the PilZ domain (Amikam and Galperin, 2006; Karaolis *et al.*, 2005). This suggests that c-di-GMP signaling is a trait that is exclusive for bacteria. Therefore, cellulose synthesis in eukaryotes must be regulated in a different way. In secondary cell-wall synthesis in plants, NAC and MYB family transcription factors have been shown to be key players. Deletion or overexpression of these transcription factors generally leads to loss or low levels of secondary cell-wall formation and ectopic secondary cell-wall deposition, respectively (Taylor, 2008 and references therein).

Phosphorylation sites have been identified for a number of *Arapidopsis* CesA proteins. Phosphorylation may target the proteins for degradation (Taylor, 2007). This is further supported by the finding that

the half-life of a CesA protein in cellulose producing cotton cells is less than 30 min, which is much shorter than for the average membrane protein (Jacob-Wilk *et al.*, 2006). This phosphorylation signal could be part of a feedback communication with the cell wall, where the cellulose is deposited. This could be achieved through the action of membrane-spanning receptor-like kinases, which trigger a kinase cascade (Taylor, 2008).

11.5 Concluding remarks

The increasing global demand for efficiently and sustainably produced forms of transport fuel and feedstock chemicals provides ample opportunity to further develop the photanol concept to products beyond the short-chain alcohols. The detailed knowledge already available about cellulose synthases and their regulation, plus emerging insights into the process of cellulose secretion in cyanobacteria make cellulose to an attractive polymer for the application of the photanol concept in an economically viable production process. The toolbox of classical physiology, combined with synthetic biology, provides ample opportunities to take up this challenge.

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Chapter 12

Exopolysaccharides from cyanobacteria and their possible industrial applications

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12.1 Introduction

In the last sixty years, a number of papers dedicated to the exopolysaccharides (EPSs) produced by cyanobacteria have been published and data on the characteristics of more than 160 polymers produced by strains of various genera and species are currently available (Pereira *et al*., 2009). The majority of these papers are on the chemical characteristics of the polymers, which possess very peculiar features in comparison with other microbial polysaccharides, and also on the factors affecting their synthesis. However, only a limited number of papers assessing the possible industrial applications of cyanobacterial EPSs (CEPSs) are available. The interest in CEPSs is largely because they may be a good alternative to polysaccharides of plant and macroalgal origin. Cyanobacteria could produce large amount of EPSs due their much higher growth rates.

Based on their thickness, consistency, and appearance, the polysaccharidic investments synthesized by cyanobacteria have been classified into three different types: sheaths, capsules, and slimes (De Philippis and Vincenzini, 1998; 2003). The sheath is a thin dense layer that loosely surrounds cells or cell groups, and is usually visible using light microscopy without staining. The capsule generally consists of a thick and slimy layer intimately associated with the cell surface, is structurally coherent, and capable of excluding particles such as India ink. The slime is a mucilaginous material dispersed around the cells without reflecting their shape. In all the three cases, aliquots of the EPS can be released and solubilized in the culture medium. This soluble polysaccharide has been termed "released polysaccharide" (RPS).

RPS is also derived from a biosynthetic process not directly related to the synthesis of EPS. In this case, the

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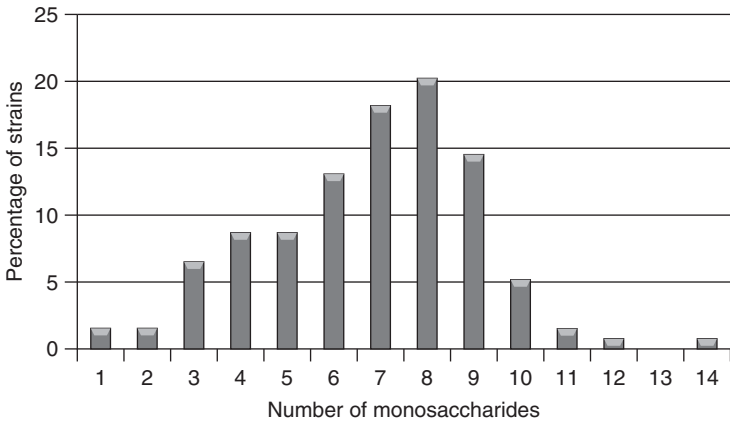

Figure 12.1 Cyanobacterial strains (percentage of total strains investigated) sharing the number of different monosaccharides in the released polysaccharide.

synthesis and the release of the polymer occur even in absence of the above-mentioned extracellular investments. The differences observed in the sulfur content and in the monosaccharidic composition of the sheath and of the RPS of some cyanobacteria strongly support the hypothesis of the presence of different biosynthetic pathways in these microorganisms (Tease *et al.*, 1991; Ortega-Calvo and Stal, 1994; Li, Harding, and Liu, 2002; Micheletti *et al.*, 2008b). Owing to their presence as solute in the culture medium, cyanobacterial RPSs can be easily recovered from liquid cultures, and for this reason they have been considered most suitable for a variety of industrial applications (De Philippis and Vincenzini, 1998; 2003).

The growth conditions (e.g., nutrient composition), environmental factors (e.g., temperature or water availability), and growth phase may affect the composition of the CEPSs (De Philippis and Vincenzini, 1998; De Vuyst and Degeest, 1999; Li, Harding, and Liu, 2002; Ricciardi *et al.*, 2002; Fischer, Marioli, and Mori, 2003; Bahat-Samet, Castro-Sowinski, and Okon, 2004).

The polysaccharides synthesized by cyanobacteria are complex heteropolymers composed of 2–14 different kinds of monosaccharides (De Philippis and Vincenzini, 1998; Sutherland, 2001; Pereira *et al.*, 2009). Only two cyanobacterial homopolymers (i.e., polysaccharides composed of only one kind of monosaccharide) have so far been described (Forni, Telo, and Grilli Caiola, 1997; Chi, Su, and Lu, 2007). Nearly 75% of the CEPSs are composed of six or more different monosaccharides (Figure 12.1). The different kinds (eleven in number) of monosaccharides identified in the CEPSs, are (De Philippis and Vincenzini, 1998, 2003; De Philippis *et al.*, 2001):

- hexoses (glucose, galactose, mannose and fructose)
- pentoses (ribose, xylose and arabinose)
- deoxyhexoses (fucose and rhamnose)
- acidic hexoses (glucuronic and galacturonic acids).

Some additional types of monosaccharides (i.e., methyl sugars and/or amino sugars) have also been identified, but only in a limited number of CEPSs (Hu *et al.*, 2003).

The high numbers of monosaccharides are peculiar to CEPSs. The polymers synthesized by macroalgae and other bacteria contain low numbers of different monomers, usually less than four (De Philippis and Vincenzini, 1998). Furthermore, the presence of peptidic and acetylated amino sugar moieties and of other non-carbohydrate constituents (e.g., phosphate, pyruvate, lactate, acetate, and glycerol) has been reported for a large number of polymers, which increases their complexity (De Vuyst and Degeest, 1999; De Vuyst *et al.*, 2001; Ruas-Madiedo, Hugenholtz, and Zoon, 2002; Girard and Schaffer-Lequart, 2007).

From the data available on the monosaccharidic composition of CEPSs, it is possible to deduce some peculiar features of these polymers in comparison with those produced by other microorganisms. Nearly 90%

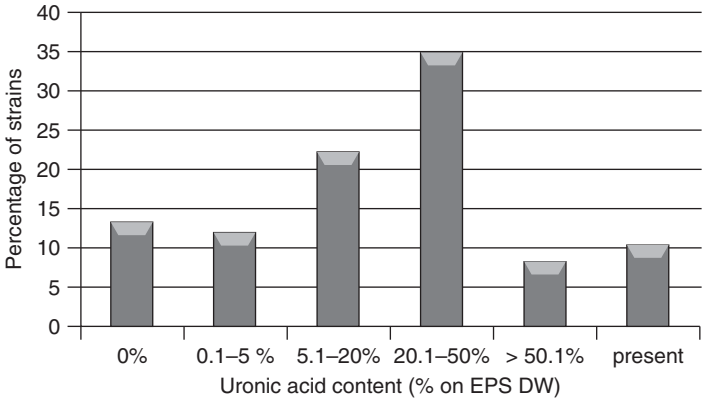

Figure 12.2 Cyanobacterial strains (percentage of total strains investigated) sharing the uronic acid content in the released polysaccharide (present = uronic acids found but not quantified; 0%, uronic acids not found).

of them have an anionic nature due to the presence of one or two uronic acids (Figure 12.2). The anionic nature could also be due to the presence of sulfate groups, a feature observed in the EPS produced by archaea and eukaryotes, but never found in other bacteria. Besides contributing to the anionic nature of the EPS, these two negatively charged groups also confer sticky behavior to the macromolecule (Decho, 1990; Sutherland, 1996; Leppard, Heissenberg, and Herndl, 1996; Arias *et al.*, 2003; De Philippis and Vincenzini, 2003; Mancuso Nichols, Guezennec, and Bowman, 2005). Moreover, their negative charge results in high affinity of CEPSs toward metal cations. In recent years, interest in the use of EPS-producing cyanobacterial strains for the biosorption of heavy metals has increased considerably (Brown and Lester, 1982; De Philippis *et al.*, 2000; Mancuso Nichols, Guezennec, and Bowman, 2005; Micheletti *et al.*, 2008b; De Philippis, Colica, and Micheletti, 2011). Moreover, the presence of ester-linked acetyl groups (up to 12% of EPS dry weight), peptidic moieties, and deoxysugars such as fucose and rhamnose in CEPSs is responsible for their hydrophobicity (Pereira *et al.*, 2009). These hydrophobic groups contribute to the emulsifying properties of the polysaccharides, which would otherwise be highly hydrophilic, and also to their rheological properties (Neu *et al.*, 1992; Shepherd *et al.*, 1995). The dual nature (hydrophobic and hydrophilic) of the polysaccharidic matrix enables cyanobacterial biofilms to attach to stone surfaces while maintaining stability against wind and water erosion (Rossi *et al.*, 2012b).

The chemical properties of CEPSs can potentially be easily modified through genetic engineering in the producing organism (Selbmann *et al.*, 2002; Parikh and Madamwar, 2006). However, no such attempt has so far been made, probably due to difficulties deriving from the large number of genes involved in the complex biosynthetic pathway (Pereira *et al.*, 2009).

In this chapter, we review studies envisaging the possible exploitation of EPS-producing cyanobacteria, with emphasis on those applications that are closer to real industrial or practical exploitation.

12.2 Potential applications of cyanobacterial exopolysaccharides

12.2.1 CEPSs as thickening or suspending agents

The features of CEPSs that at first attracted the attention of researchers for possible industrial exploitation were the high viscosity of their aqueous solutions, capability to form gels with good tensile strength, and capability to stabilize emulsions. In addition to composition and structure (Margaritis and Pace, 1985), a feature that determines the rheological behavior of the aqueous solutions of polysaccharides is their

high molecular weight (MW) (Shepherd *et al.*, 1995; Kamal *et al.*, 2003), ranging from 0.1 to about 2 MDa (Pereira *et al.*, 2009). The highest MW values (about 2 MDa) reported were for the EPSs synthesized by *Anabaena spiroides* (Colombo, Vieira, and Moraes, 2004), *Cyanospira capsulata* ATCC 43193 (Vincenzini *et al.*, 1993), and *Phormidium* 94a (Vicente-Garcia *et al.*, 2004). These values are almost twice the molecular mass of xanthan gum, the microbial polysaccharide most frequently utilized for industrial applications, which has molecular mass of about 1 MDa.

Despite these promising features, the rheology of CEPSs is poorly investigated. Most studies deal with the dependence of viscosity on the shear rate of CRPSs aqueous solutions (De Philippis and Vincenzini, 1998). The most important industrial property is their ability to stabilize the flow properties of aqueous solutions against changes in temperature, ionic strength, and pH (Sutherland, 1999, De Vuyst and Degeest, 1999). The flow properties of some CRPSs have shown viscosity values comparable to, or even better than, those of aqueous solutions of xanthan gum, at comparable concentrations. Moreover, they show an appreciable stability of the viscosity over a wide range of pHs, temperatures, and sodium chloride concentrations (Lapasin *et al.*, 1992; De Philippis *et al.*, 2000; Moreno *et al.*, 2000; Morris *et al.*, 2001). The presence of both hydrophilic and hydrophobic groups in the CRPSs suggests a possible use in the stabilization of emulsions or as bioflocculants.

Because of their inability to compete in the market with xanthan gum (the most utilized microbial polysaccharide), CEPSs have failed to make a mark in commercial markets. Firstly, the industrial production of xanthan gum is a well-consolidated process, while CEPSs production is time-consuming and expensive. Secondly, xanthan gum has been registered as a food additive with the US Food and Drug Administration and the European Union for more than 15 years (Sutherland, 1996), while any new polysaccharide has to go through a long and expensive process before it can be commercialized. Consequently, only polymers possessing new and unique properties will be considered for such a complex process. Thus only a few new microbial polysaccharides have entered the market (e.g., curdlan, dextran, and gellan) (Rhem, 2010).

12.2.2 CEPSs as antiviral agents

The application of CEPSs that has attracted most attention in the last few years relates with their potential antiviral activity, which is mainly due to the presence of sulfate groups in the CEPSs, often reaching about 20% of EPS dry weight (Liu and Thorp, 2002; Pujol *et al.*, 2007; Pereira *et al.*, 2009).

Lau *et al.* (1993) reported that certain cyanobacteria inhibit the reverse transcriptase of the HIV and avian myeloblastosis virus, due to a sulfolipid secreted into the extracellular medium by the cyanobacteria (Gustafson *et al.*, 1989; Sudo *et al.*, 1995). Other studies reporting antiviral activity in CEPSs are Hayashi, Hayashi and Kojima (1996); Hayashi *et al.* (1996); Schaeffer and Krylov (2000); Cordova *et al.* (2002); Ascencio *et al.* (2004); Kanekiyo *et al.* (2005); Balachandran *et al.* (2006); Grzanna *et al.* (2006); and Kanekiyo *et al.* (2007). More recently, Lobner *et al.* (2008) investigated the effect of Immulina, a high-molecular-weight polysaccharide extracted from *Arthrospira platensis*, on adaptive immune responses and demonstrated that the polysaccharide caused an age-dependent, temporary enhancement of adaptive immune responses. Radonic *et al.* (2010) showed that the sulfated exopolysaccharide (TK V3) isolated from *Arthrospira platensis* acts as an inhibitor of orthopoxvirus and other enveloped viruses.

The features that are considered essential for conferring good antiviral properties to a polysaccharide are MW in the range 10–100 kDa (Damonte, Matulewicz, and Cerezo, 2004); sulfate content of 20% or more; good distribution of sulfate groups in the macromolecule; macromolecule shape suitable for good adhesion to the virus surface; and the presence of hydrophobic groups on the macromolecule, which is considered important for adhesion to viral proteins (Pujol *et al.*, 2007; Ghosh *et al.*, 2009). The direct use of CEPSs with high percentages of sulfate and hydrophobic groups as antiviral agents is limited by the high MW of these polymers (Pereira *et al.*, 2009), and they can therefore only be used as antiviral agents after being subjected to a mild hydrolysis, carried out to obtain oligosaccharides with a MW in the range 10–100 kDa.

12.2.3 Use of CEPSs in cosmetics

The presence of charged groups (confers capability to bind water molecules) in CEPSs makes them valuable for cosmetics (Sutherland, 1994). Moreover, accumulation of scytonemin and mycosporine-like pigments in the external polysaccharidic layers of some cyanobacteria, which protect them against dangerous high-energy UV radiation (Pattainak, Schuman and Karsten, 2007; Rossi *et al.*, 2012b), suggests the possibility of using the extracted polysaccharides in cosmetics (sun-tan and skin-protection lotions). A detailed account of cyanobacterial photoprotective compounds has been provided in Chapter 8.

However, studies on real applications are few, with the exception of a recent US patent. In the patent, an exopolysaccharide extracted from a microbial mat found in French Polynesia was reported to be useful in reducing and preventing skin aging and environmental damage. The effect was achieved by altering skin-cell metabolism and improving hydration (Loing *et al.*, 2011), but the idea is yet to be exploited industrially.

12.2.4 CEPSs in prevention of the erosion of desert soils

The exploitation of microalgae and cyanobacteria that release large amounts of exopolysaccharides has shown promising results in the prevention of desertification (Chen *et al.*, 2006; Wang *et al.*, 2009). Some cyanobacteria show polyextremophilic characteristics (e.g., tolerance to desiccation and to high solar irradiation) because of their ability to produce large amounts of EPSs and to fix nitrogen. These characteristics help cyanobacteria to retain the limited amount of water available in desert areas. Sub-surface cyanobacteria (together with other microalgae and fungi) that live a few millimeters beneath the desert surface not only influence the behavior of water in the soil but also contribute greatly to nitrogen and carbon fixation *in situ* (Acea, Diz, and Prieto-Fernandez, 2003; Belnap 2006). Microalgal and cyanobacterial desert crusts are abundant in filamentous–sheath-forming and EPS-excreting cyanobacteria (Mazor *et al.*, 1996). Unlike transient rain crusts, they are critical to the eco-environments in which they occur. The prevalence of cyanobacterial soil crusts helps in maintaining surface stability and in decreasing erosion in dryland soils (Thomas and Dougill, 2007); survival of cyanobacterial soil crusts is related to their capability to produce EPSs.

CEPSs stabilize degraded soils by gluing soil particles and enhance fertility by supplying biologically usable nitrogen (Nisha, Kaushik, and Kaushik, 2007; Su *et al.*, 2011; Rossi *et al.*, 2012a). The EPS matrix is capable of both physical and chemical immobilization of nutrients, maintaining them in the soil crust and acting as a reservoir, which can be used by the microflora residing in the crust (Hawkes 2003; Veluci, Neher, and Weicht, 2006). Although biological soil crusts (BSCs) may increase nitrogen input into the soil through fixation, much of this nitrogen is released immediately into the surrounding soil (Belnap, 2003), suggesting either the loss of fixed nitrogen to the atmosphere or its leaching (Mager and Thomas, 2011). Similarly, secreted CEPSs form a mechanical structure surrounding the cells, which together with soil particles form a heterogeneous mass or stable aggregate in the topsoil (Mazor *et al.*, 1996), decreasing carbon loss by erosion (Mager and Thomas, 2011).

The feasibility of the inoculation of cyanobacteria for inducing the formation of soil crusts to stabilize sand dunes in desert areas has been assessed. Chen *et al.* (2006) showed that BSCs formed after the inoculation of cyanobacteria on the sand are effective in increasing the carbon and nitrogen content of the soil and in promoting the growth of herbaceous and subshrub cover on the dunes. As a consequence, a higher resistance of the soil to erosion was observed, leading to long-term fixation of unconsolidated sand. In field experiments carried out in Hobq Desert (Inner Mongolia, China), Wang *et al.* (2009) showed that after inoculation, in some areas, cyanobacterial and algal cover reached 48.5% and cyanobacterial inoculation increased organic carbon and total nitrogen in the soil. Xu *et al.* (2013) reported that the presence of *Phormidium tenue*, a polysaccharide-excreting cyanobacterium dominant in BSCs, in the Tengger Desert (China) promoted the growth of a dominant desert sub-shrub by increasing seed germination rates, ion uptake, and photosynthetic activity.

The use of microbiotic crust cultivation therefore constitutes a promising field of practical application of these microorganisms. In particular, they can be utilized for stabilizing unconsolidated soil particles and for fixing moving dunes to fight desertification and prevent sandstorms.

12.2.5 EPS-producing cyanobacteria in metal biosorption

12.2.5.1 Metal biosorption by EPS-producing cyanobacteria

The use of microorganisms for the removal of toxic heavy metals from polluted waters has been intensively investigated since the 1980s and the efficiency in metal chelation of a number of them has been assessed. The presence of a high number of negatively charged groups on the cell envelope is the main reason for this efficiency (Gadd, 2009).

The presence of a large number of negative charges on the external cell layers of EPS-producing cyanobacteria (Pereira *et al*., 2009) suggests their use as chelating agents for the removal of positively charged heavy metal ions from water solutions (De Philippis and Micheletti, 2009) and a large number of studies on the use of cyanobacteria in metal biosorption have been recently published (De Philippis, Colica, and Micheletti, 2011).

A clear demonstration of the role played by the EPS in the metal uptake was given by De Philippis, Paperi, and Sili (2007), who compared the biosorption performances obtained with the biomass of two capsulated cyanobacteria (De Philippis *et al*., 2003) with those obtained with whole cultures of the same cyanobacteria. The differences observed in the metal uptake performances in the two systems were quite congruent with the specific metal removal of solutions of the pure exopolysaccharides obtained from the cultures of the two cyanobacteria, pointing to the role of the polymer in solution in the biosorption process.

The significant contribution of the sheath in the metal-binding process has also been demonstrated, by comparing the specific metal uptake of the sheathed cyanobacterium *Gloeothece* PCC 6909 and of its sheathless mutant PCC 6909/1 (Micheletti *et al*., 2008b). Many studies have shown the role of the negative charges generally present in the CEPSs in the metal sorbing performances of EPS-producing cyanobacteria (Micheletti *et al*., 2008a,2008b; Pereira *et al*., 2009; De Philippis, Colica, and Micheletti, 2011).

12.2.5.2 CEPSs in metal bioremoval from industrial wastewaters

Studies have shown the use of EPS-producing cyanobacteria for metal biosorption from wastewaters on large scales. In nature the sorption process is much more complex than under laboratory conditions for a number of reasons:

- the concentration and the variety of the chemical species of metals present in wastewaters
- the presence of additional organic and inorganic compounds in the effluent
- the value of pH, often far from the optimal value for biosorption
- limited solubility of the metal to be removed due to its presence as suspended solid particles or complexed with other compounds
- the temperature of the effluent and the anisothermic conditions of the process
- the type, dimension, and geometry of the devices used for the containment of the microbial biomass.

Thus frequent maintenance of the operational conditions is critical for the retention of the biosorption performance observed under laboratory conditions with pure metal solutions.

The way cyanobacteria are put in contact with the metal-containing wastewaters is one of the most critical issues in the use of microbial biosorbents. In most cases, biosorbents have been prepared in grains, making the system quite similar to the ion-exchange resins used in columns (Davis, Volesky, and Mucci, 2003; Volesky, 2007; Kiran and Kaushik, 2008). Another option is the use of microbial cells immobilized in the form of biofilms grown on solid supports and utilized in rotating plates, fixed-bed reactors, trickle filters, fluidized beds, and airlift bioreactors (Gadd, 2009). Dead and/or living microbial biomass immobilized with different chemical compounds have been used, for example polyacrylamide, toluene diisocyanate, glutaraldehyde, agar, cellulose acetate, alginate, polyurethane foam blocks, glass beads, and carrageenan (Nakajima, Horikoshi, and Sakaguchi, 1982).

Due to their ability to form resistant biofilms, EPS-producing cyanobacteria are considered a promising group of microorganisms for metal biosorption (Sutherland, 2001; Roeselers, van Loosdrecht, and Muyzer, 2008). Biofilms formed by them show good resistance to detachment (Roeselers, van Loosdrecht, and Muyzer, 2008, Gadd, 2009; Kumar, Pandey, and Gaur, 2010). The EPS-producing cyanobacterium *Nostoc* PCC7936, confined in three different filtering systems, was used at a pilot scale for the treatment of industrial wastewater containing chromium (VI) (Colica, Mecarozzi, and De Philippis, 2010). The results were very promising in comparison with those obtained with other microorganisms utilized for chromium removal (Kratochvil, Pimentel, and Volesky, 1998; Dakiky *et al.*, 2002; Arica *et al.*, 2005), and demonstrated that flow-through systems give better results than batch systems. However, there is a need to maintain as much EPS as possible into the flow-through device, as this fraction is responsible for the removal of chromium (III) formed due to the oxidation of the biomass carried out by chromium (VI).

However, to date no industrial-scale process has been developed, mainly due to the high costs of the production, harvesting, and preparation of the biomass used in the process. To reduce the costs of the biosorption, it has been suggested that the cyanobacterial biomass could be used for several consecutive sorbing–desorbing cycles by incorporating a non-destructive recovery of the microorganisms (Paperi, Micheletti, and De Philippis, 2006; Volesky, 2007; Gadd, 2009; Chojnacka, 2010). The recovery of commercially valuable metals from the biomass (Gadd, 2009, Machado, Soares and Soares, 2010) has also been proposed, counterbalancing the costs of production with revenue derived from the sale of the recovered metals, especially precious metals such as gold. The experiments carried out for recovering gold from industrial wastewaters have shown promising results (Colica *et al.*, 2012).

12.3 Concluding remarks

Despite the promising results and good exploitation perspectives, no CEPSs-based product or process have been commercial exploited. A number of explanations can be given:

- the cost of growing the cyanobacteria is quite high and is a major obstacle to their exploitation for obtaining products that can be cheaply obtained from other industrial processes
- lack of clear demonstrations of results carried out at pilot scale, as well as the advantages of use of CEPSs
- lack of industrial support for the research involved in identifying new polysaccharides for new technological applications
- lack of industrial-scale cultivation of EPS-producing cyanobacteria, a process that is expected to be quite complex owing to the progressive increase of culture viscosity due to the presence of CEPSs.

However, there are many reasons to believe that in the future cyanobacteria will be utilized for industrial-scale polysaccharide production:

- the large number of strains capable of producing EPSs with very diverse chemical and macromolecular properties, a feature that suggests a good chance of finding polymers possessing specific properties for applications not covered by the macromolecules already on the market
- increases in the prices of the chemical products, which progressively reduce the difference in costs between chemical and biological processes for obtaining polymers of industrial interest
- increasing public perception of the use of green biotechnologies to obtain industrial products.

However, to optimize production, and the chemical and macromolecular characteristics, as well as to make CEPSs economically competitive with the polysaccharides obtained from other sources, we need to enhance our understanding of the metabolism of EPS-producing cyanobacteria, and in particular the genes involved in the biosynthetic pathways of these polymers.

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Chapter 13
Phycocyanins

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13.1 Introduction

Phycobiliproteins are proteins that form light-harvesting antenna complexes (phycobilisomes) and act as photosynthetic accessory pigments in cyanobacteria (blue-green algae), in Rhodophyta (the red algae), and in Cryptophyta (cryptomonads). The cyanobacteria are an extraordinarily diverse group of photoautotrophic prokaryotes, growing in a wide range of habitats. Since the majority of cyanobacterial species are obligate phototropic, they are normally restricted to light environments. Many cyanobacterial species can be readily grown in mass culture, with the highest amounts of phycobiliproteins (PCBs) reported in organisms cultured in white light of low intensity.

PCBs are proteins with covalently attached linear tetrapyrrole prosthetic groups called bilins. Their conformational and steric constraints produce their interesting spectroscopic properties. These macromolecules are a family of hydrophilic, brilliantly colored, and stable fluorescent pigment proteins. PCBs absorb visible light from 450–650 nm and are divided into four classes based on their absorption spectra: phycoerythrins (PEs, $\lambda_{max} \sim 540–570$ nm, appear red), phycocyanins (PCs, $\lambda_{max} \sim 610–620$ nm), phycoerythrocyanins (PECs, $\lambda_{max} \sim 550$ nm) and allophycocyanins (APCs, $\lambda_{max} \sim 650–655$ nm) (Glazer, 1999).

Owing to their excellent spectroscopic properties, stability, high absorption coefficients, and high quantum yields, PCBs have a wide range of promising applications in biomedical research, diagnostics, and therapeutics (Kronic and Grossman, 1983; Glazer, 1984, 1994; Chowdhury *et al.*, 2007). In addition, they have potential as natural colorants for use in food, cosmetics, and pharmaceuticals, particularly as substitutes for synthetic dyes, which are generally toxic or otherwise unsafe (Arad and Yaron, 1992; Bermejo *et al.*, 2003a; Eriksen, 2008). There are approximately 60 patents on PCB production, 30 patents on applications in medicine, food, and others areas, and 240 patents on applications utilizing the fluorescence properties of PCBs (Sekar and Chandramohan, 2008).

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13.2 Phycocyanins

Phycocyanins (PCs) are water-soluble phycobiliproteins, with extraordinary fluorescent properties (high quantum yield, high Stokes shift, and an important insensitivity to quenching). They have therapeutic value due to their protective effect against various conditions and their anticarcinogenic activity (Remirez *et al.*, 1999; Rimbau *et al.*, 1999), their antioxidative and anti-inflammatory activities (Bhat and Madyastha, 2000; Benedetti *et al.*, 2004), and in the treatment of Alzheimer's and Parkinson's disease (Rimbau *et al.*, 2001). In terms of their absorption properties, PCs are divided into three main classes: C-phycocyanin (C-PC, $\lambda_{max} \sim 610–620$ nm), allophycocyanin (APC, $\lambda_{max} \sim 650–655$ nm) and R-phycocyanin (R-PC, $\lambda_{max} \sim 550$, 618 nm). Some cyanobacteria have a fourth type of phycocyanin, the phycoerythrocyanin (PEC, $\lambda_{max} \sim 550$ nm). Visually, PCs' color range encompasses purple (PEC, R-PC) and deep blue (C-PC), while APC is blue with a hint of green.

13.2.1 Subunit composition

PCs are composed of two relatively homologous subunits: the α and β chains. Both chains are rich in conserved α-helices, and the overall three-dimensional subunit structures are similar in different organisms. Large amino-acid-sequence similarities exist between the same types of PC from all cyanobacteria and red algae (Adir, Dobrovetsky, and Lerner, 2001; Contreras-Martel *et al.*, 2007). Table 13.1 shows the amino acid sequences of α and β subunits for C-PC and APC from *Spirulina platensis*, R-PC from *Porphyridium cruentum*, and PEC from *Mastigocladus laminosus*.

For each of the different types of PC, the building block is an $\alpha\beta$ heterodimer. The masses of α and β subunits range between 18 and 22 kDa. The heterodimer is a very stable unit, which resists dissociation (protein concentration as low as 10^{-12} M) in the presence of chaotropic agents (MacColl *et al.*, 1981). Table 13.2 shows the molecular weights for α and β subunits from different PCs.

13.2.2 Bilin composition

Three isomeric bilins – phycocyanobilin (PCB), phycobiliviolin (PVB), and phycoerythrobilin (PEB) – are found attached to phycocyanins (Figure 13.1). These bilins are linked to the polypeptide through thioether bonds to cysteinyl residues. Figure 13.2 shows the structure of the polypeptide-bound bilin, using as example PCB linked to an apoprotein chain through a cysteinyl residue.

In native PCs, PCB gives rise to an absorbance peak at 590–625 nm, PVB at 568 nm, and PEB at 540–565 nm. The sequences of many PCs have been determined either by amino-acid sequence analysis, or by determination of the DNA sequences of the genes encoding these proteins (Sidler, 1994). Extensive studies have established the locations of the cysteinyl residues that serve as sites of bilin attachment and the identity of the bilin at each of these locations.

Table 13.3 shows bilin types and sites of attachment in PCs. C-PC carries one PCB on the α subunit and two on the β subunit. APC carries one PCB on each α and β subunit. R-PC carries one PCB bilin on the α subunit and two different bilins (PCB and PEB) on the β subunit. PEC carries one PVB bilin on the α subunit and two PCB bilins on the β subunit.

13.2.3 Oligomerization state

In cyanobacteria and red algae, PCBs in general and PCs in particular are components of large complexes called phycobilisomes that are attached to the cytoplasmatic surface of the photosynthetic lamellae. The phycobilisome is made up of disk-shaped trimeric $(\alpha\beta)_3$, and hexameric $(\alpha\beta)_6$, complexes of phycobiliproteins. The hexameric complexes are built by face-to-face interaction of the trimers.

The stability of the trimers and hexamers varies markedly with the organism and/or the phycobiliproteins, but these oligomers are less stable than the heterodimer. Thus, when PCs are released by cell breakage and subjected to the conventional procedures for protein purification, the oligomerization state of the

Table 13.1 Complete amino acid sequences of phycocyanin subunits from different cyanobacteria: C-PC and APC from *Spirulina platensis*, R-PC from *Porphyridium cruentum*, and PEC from *Mastigocladus laminosus*.

	α subunit	**β subunit**
APC	10 20 30 40 MSIVTKSIVN ADAEARYLSP GELDRIKSFV TSGERRVRIA 50 60 ETMTGARERI IKEAGNQLFQ 70 80 90 100 KRPDVVSPGG NAYGEEMTAT CLRDLDYYLR LITYGIVAGD 110 VTPIEEIGVV 120 130 140 150 GVREMYKSLG TPIEAVAEGV RAMKSVATSL LSGEDAAEAG 160 AYFDYLIGAM S	10 20 30 40 MQDAITSVIN SSDVQGKYLD RSAIQKLKAY FATGELRVRA 50 60 ATTISANAAN IVKEAVAKSL 70 80 90 100 LYSDITRPGG NMYTTRRYAA CIRDLDYYLR YATYAMLAGD 110 PSILDERVLN 120 130 140 150 GLKETYNSLG VPIGATVQAI QAMKEVTAGL VGADAGKEMG 160 IYFDYICSGL S
C-PC	10 20 30 40 MKTPLTEAVS IADSQGRFLS STEIQVAFGR FRQAKAGLEA 50 60 AKALTSKADS LISGAAQAVY 70 80 90 100 NKFPYTTQMQ GPNYAADQRG KDKCARDIGY YLRMVTYCLI 110 AGGTGPMDEY 120 130 140 150 LIAGIDEINR TFELSPSWYI EALKYIKANH GLSGDAATEA 160 NSYLDYAINA LS	10 20 30 40 MFDAFTKVVS QADTRGEMLS TAQIDALSQM VAESNKRLDA 50 60 VNRITSNAST IVSNAARSLF 70 80 90 100 AEQPQLIAPG GNAYTSRRMA ACLRDMEIIL RYVTYAVFAG 110 DASVLEDRCL NGLRE 120 130 140 150 TYLAL GTPGSSVAVG VGKMKEAALA IVNDPAGITP 160 170 GDCSALASEI ASYFDRACAA VS
R-PC	10 20 30 40 MKTPITEAIA TADNQGRFLS NTELQAVNGR YQRATASLTA 50 60 AKALTGSAQR LITGAAQAVY 70 80 90 100 NKFPYTTQMP GPAYASSAIG KAKCARDIGY YLRMVTYTLV 110 VGGTGPMDEY 120 130 140 150 LVAGLEEINR SFDLSPSWYI EALQYIKNSH GLSGQVANEA 160 NAYIDYAINT SL	10 20 30 40 MLDAFAKVVA QADARGEFLS NTQIDALSKM VKEGNQRLDI 50 60 VNKVTSNASA IVTNSARALF 70 80 90 100 AEQPQLIQPG GNAYTSRNMA ACLRDMEIVL RYVSYAMLAG 110 DSSVLDDRCL NGLRE 120 130 140 150 TYQAL GTPGSSVAVA IQKMKDASVA LANDTTGTPI 160 170 GDCSSLVAEL AGYFDRAAVS VV
PEC	10 20 30 40 MKTPLTEAIA AADLRGSYLS NTELQAVFGR FNRARAGLEA 50 60 ARAFANNGKK WAEAAANHVY 70 80 90 100 QKFPYTTQMQ GPQYASTPEG KAKCVRDIDH YLRTISYCCV 110 120 VGGTGPLDDY VVAGLKEFNS 130 140 150 160 ALGLSPSWYI AALEFVRDNH GLTGDVAGEA NTYINYAINA LS	10 20 30 40 MLDAFSRVVE QADKKGAYLS NDEINALQAIV ADSNKRLDVV 50 60 NRLTSNASSI VANAYRALV 70 80 90 100 AERPQVFNPG GPCFHHRNQA ACIRDLGFIL RYVTYSVLAG 110 120 DTSVMDDRCLNGLRETYQAL 130 140 150 160 GTPGDAVASG IKKMKEAALK IANDPNGITK GDCSQLMSEL 170 ASYFDRAAAA VA

Table 13.2 Molecular weights of phycocyanin subunits from different methodologies and authors.

Phycocyanin	α subunit (Da)	β subunit (Da)	Microalga/author
C-PC	18188	19266	*Spirulina platensis* (Padyana *et al.*, 2001)
	18092	19426	*Galdieria sulphuraria* (Troxler *et al.*, 1979, 1981)
	18000	19400	*Mastigocladus laminosus* (Frank *et al.*, 1978)
	17600	20500	*Anabaena marina* (Ramos *et al.*, 2010)
	17783	20100	*Synechocystis aquatilis* (Ramos *et al.*, 2011)
	17783	20893	*Spirulina platensis* (Bermejo and Ramos, 2012)
A-PC	17978	18001	*Spirulina platensis* (Brejc *et al.*, 1995)
	18095	18070	*Porphyra yezoensis* (Liu *et al.*, 1999)
	17709	17960	*Mastigocladus laminosus* (Sidler *et al.*, 1981)
	17932	17891	*Anabaena* sp. (Ducret *et al.*, 1996)
	17667	17902	*Anabaena cylindrica* (Minami, Yamada, and Hase, 1985)
R-PC	18049	19344	*Porphyridium cruentum* (Ducret *et al.*, 1994)
	18156	19173	*Polysiphonia urceolata* (Jiang *et al.*, 2001)
PEC	18200	19600	*Mastigocladus laminosus* (Fuglistaller *et al.*,1983)

$\alpha\beta$ heterodimers depends strongly on the purification conditions (Glazer, 1999). The oligomerization state of PCs in solution is very sensitive to different factors: pH, ionic strength, and protein concentration. At neutral pH and moderate ionic strength and protein concentration, the most common oligomerization state is the trimer. This form conserves the native protein conformation and therefore its spectral properties.

13.3 Spectroscopic properties

The absorbance and fluorescence maxima of PCs are given in Table 13.4. Partly due to the presence of multiple bilins, the absorbance coefficients of PCs are comparable with those of the most strongly absorbing dyes. The highest absorbance coefficients reported for dyes in the visible region of the spectrum are near to 250,000/M/cm.

Figure 13.3 shows absorption, fluorescence, and anisotropy spectra of the main PCs (C-PC, APC, and R-PC). The absorption spectra of the trimeric and monomeric C-PC forms are only slightly affected by the oligomerization changes in the protein (Saxena, 1988). On the other hand, the dissociation of trimeric APC into monomeric APC causes important absorption and fluorescence spectral changes, and because of this it is easy to distinguish the oligomerization state of APC by examining its spectrum. It is important to emphasize that the easiest and most explanatory spectroscopic test to determine the oligomerization state of PCs is to record the excitation fluorescence steady-state anisotropy spectrum. Since energy transfer between chromophores modulates the fluorescent properties of these proteins, dramatic increases in the anisotropy spectra are observed when the oligomerization state of the PCs changes from trimers to monomers, due to the chromophores uncoupling, which alters the pathways for energy transfer between them (Bermejo *et al.*, 2006).

High-resolution crystal structure determinations have been recorded for PCs (Table 13.5; Duerring *et al.*, 1990; Duerring, Schmidt and Huber, 1991; Brejc *et al.*, 1995). In the native phycocyanins, the bilins are held in extended configurations (Figure 13.4; Padyana *et al.*, 2001; David, Marx, and Adir, 2011). In this configuration, bilins exhibit the strongest absorbance at their long-wavelength maxima. When PCs are denatured by urea, sodium dodecyl sulfate, or at extremes of pH, the bilins are released from three-dimensional constraints, and assume cyclohelical conformations with a concomitant large decrease in their long-wavelength absorbance and a complete loss of their fluorescence emission (Glazer, 1986).

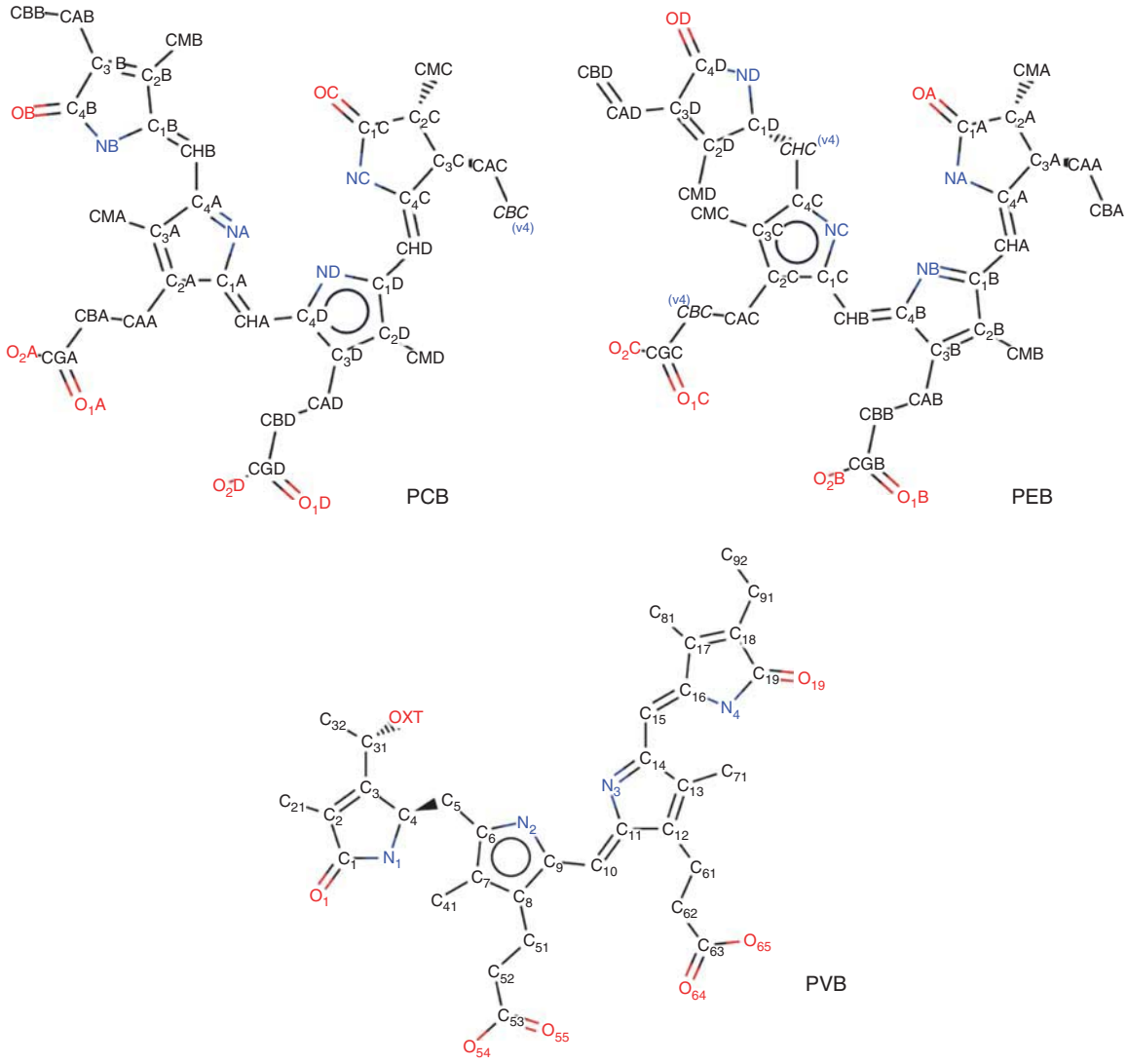

Figure 13.1 Structures of the different bilins (PCB, PEB, PVB) present in phycocyanins. See plate section for color version.

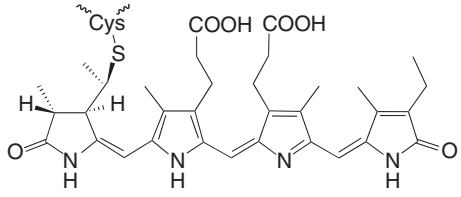

Figure 13.2 Phycocyanobilin (PCB) linked to the polypeptide through thioether bound to cysteinyl residues.

Regarding fluorescence quantum yield, the bilins in PCs are held and largely protected from the solvent, so excitation of PCs leads to fluorescence emissions with high quantum yields (Table 13.4). For this reason radiationless decay due to motion or collision with quencher molecules is minimized. In contrast, free bilins or denatured phycocyanins are virtually nonfluorescent.

Table 13.3 Bilin types and sites of attachment in phycocyanins. The designations such as "α-84", indicate the phycobiliprotein subunit and position of the cysteinyl residue that functions as the site of bilin attachment.

Phycocyanin	α subunit (aminoacids and bilins)	β subunit (aminoacids and bilins)
C-phycocyanin C-PC	α:162 Amino acids 1 PCB (α84)	β: 172 amino acids 2 PCB (β82, β155)
Allophycocyanin APC	α:161 amino acids 1 PCB (α81)	β: 161 amino acids 1PCB (β81)
R-phycocyanin R-PC	α:162 amino acids 1 PCB (α84)	β: 172 amino acids, 1 PCB (β82) 1 PEB (β155)
Phycoerythrocyanin PEC	α: 162 amino acids 1 PVB (α84)	β: 172 amino acids, 1 PCB (β82), 1 PCB (β153)

13.4 Isolation and purification procedures for phycocyanins

The widespread use of PCs and other phycobiliproteins has been somewhat limited by the high purification cost of these macromolecules, ranging from $10 to $50/mg (Prozyme, ABDBioquest Inc.). For uses as a colorant this price has gone down to $1–5/g because high purity is not required (Sigma, 2009). A simple approach to determine a PC's purity is to evaluate the absorbance ratio $A_{visible}/A_{280}$, since the visible maximum represents the specific PC concentration and the value at 280 nm represents the aromatic amino acids present in all proteins. For example, C-PC protein solutions with A_{620}/A_{280} greater than 0.7 are considered food grade, those with the ratio greater 3.5 are suitable for reactive grade, and those with ratio greater than 4.0 are suitable for analytical grade.

The conventional schemes for PC purification utilize a sequence of operations that incorporate several complex methods, such as precipitation, centrifugation, dialysis, ion exchange chromatography, gel filtration, and chromatography on hydroxyapatite. In general, conventional methodologies involve two processes: sample pretreatment to remove the intracellular material (obtaining crude extract) and purification of PCs using chromatography.

13.4.1 Extraction of phycocyanins

PCs can be extracted from cyanobacteria using different methodologies, which combine breakage of cell walls and extraction of the water-soluble PCs into aqueous media. From dried cyanobacterial biomass (depending on the species), PCs have been extracted by re-suspending the biomass in acetate or phosphate buffers with different ionic strength and pH values (Doke 2005; Bermejo *et al.*, 2006; Niu *et al.*, 2007; Oliveira *et al.*, 2008; Ramos *et al.*, 2010, 2011). From wet biomass, PCs have been extracted by subjecting the biomass to cycles of freezing to temperatures between -25 and -15°C in liquid nitrogen, and thawing to 5–30°C (Minkova *et al.*, 2003; Doke, 2005; Soni *et al.*, 2006). Compared to alternative methods, freeze/thaw cycles have proven to be the most efficient way to extract PCs from wet cyanobacterial biomass (Abalde *et al.*, 1998; Doke, 2005). There are also other

Table 13.4 Spectroscopic properties of phycocyanins. (Φ_F: fluorescence quantum yield; ε_M: molar extinction coefficient).

Phycocyanin	Molecular mass (kDa)	λ_{max} absorption	ε_M ($M^{-1}cm^{-1}$)	λ_{max} fluorescence	Φ_F*
Allophycocyanin $(\alpha\beta)_3$	100.0	650	696,000	660	0'68
C-phycocyanin $(\alpha\beta)_3$	110.1	615	281,000	642	0'51
R-phycocyanin $(\alpha\beta)_3$	140.0	550, 618	240,000	636	–
Phycoerythrocyanin $(\alpha\beta)_3$	113.4	570	606,000	640	–

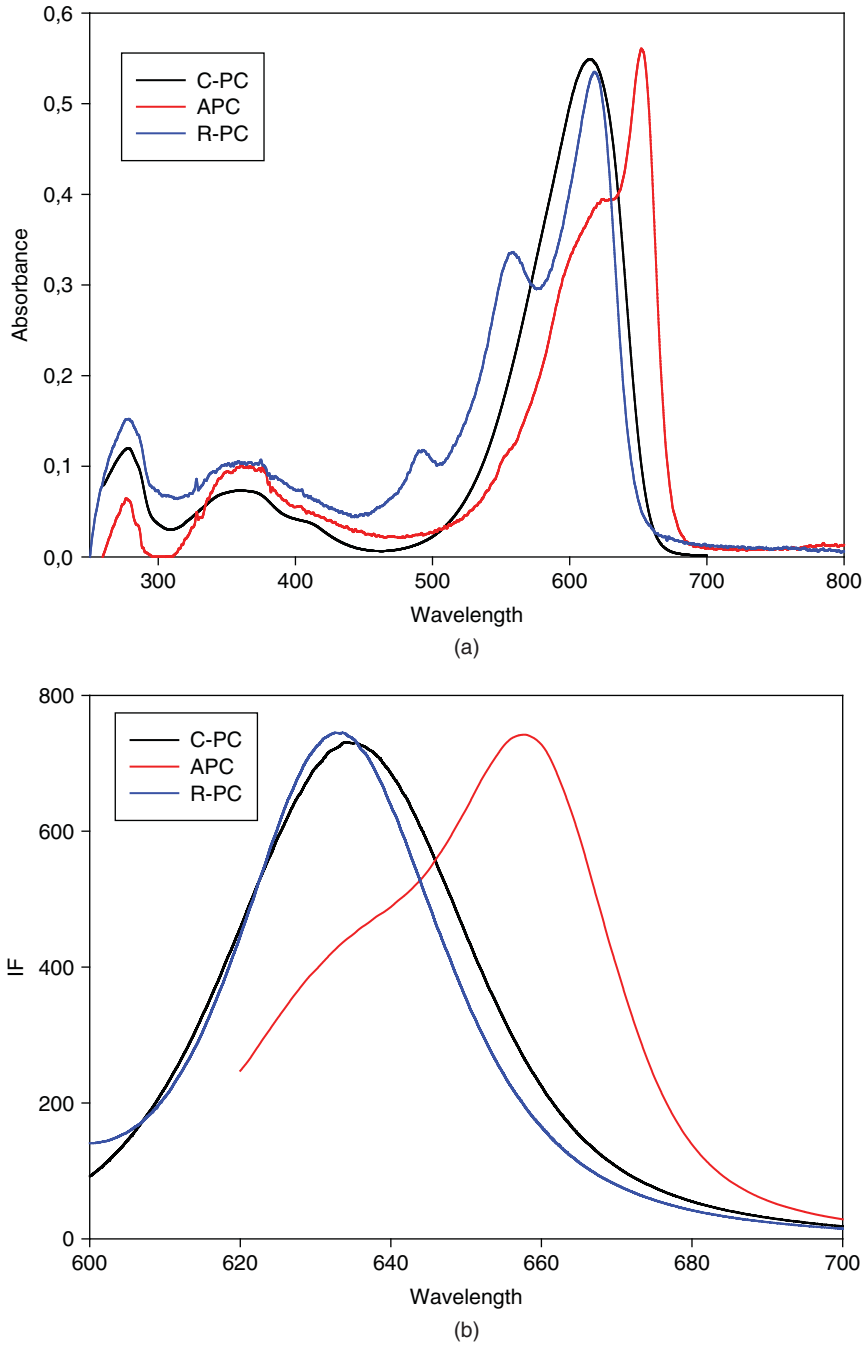

Figure 13.3 Spectroscopic characterization of C-PC (black line), APC (red line), and R-PC (blue line) in 5 mM sodium phosphate buffer, pH 7.0. (a) Absorbance spectra; (b), fluorescence emission spectra; (c) excitation fluorescence anisotropy spectra. The anisotropy values are the arithmetical average of five anisotropy measurements and the error bars are the standard deviations of these anisotropy determinations. See plate section for color version.

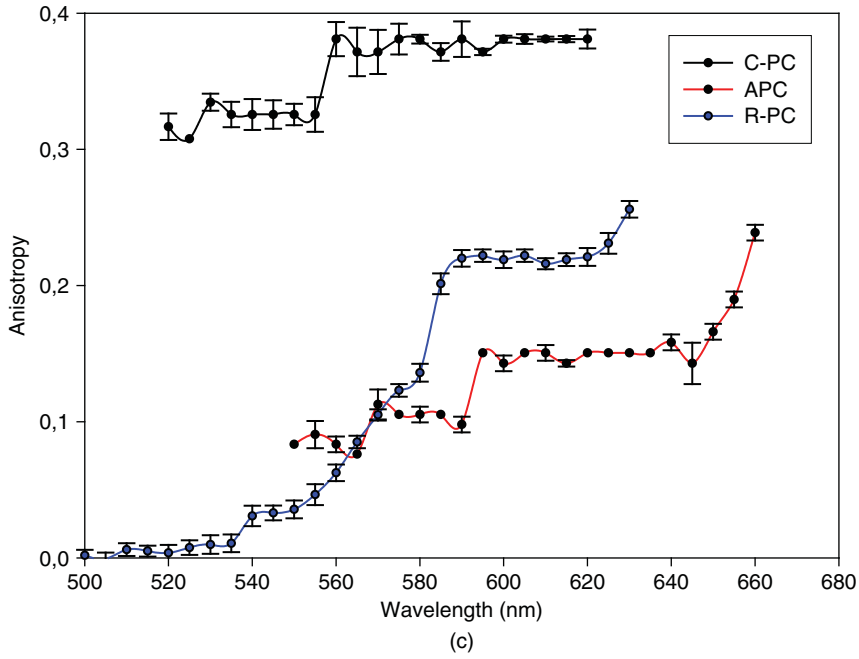

Figure 13.3 (*continued*)

Table 13.5 Crystallographic parameters from phycocyanins high-resolution crystal structures.

	Distance (Å)			Angles (Å)			Reference
Phycocyanin	**a**	**b**	**c**	**α**	**β**	**γ**	
C-PC	107.2	115.4	183.04	90.2	90.2	–	Padyana *et al.* (2001)
A-PC	101.9	101.9	130.6	90.0	90.0	120.0	Brejc *et al.* (1995)
	105.3	105.3	189.4	90.0	90.0	120.0	Liu *et al.* (1999)
R-PC	135.1	135.1	210	90.0	90.0	90.0	Jiang *et al.* (2001)
PEC	156.74	156.74	40.20	90.0	90.0	120.0	Schmidt, Krassett, and Reuter (2006)

methods to extract PCs: ultrasound (Abalde *et al.*, 1998), mechanical breakage (Doke, 2005; Schmidt, Wiebe, and Eriksen, 2005), high pressure exposure (Patil *et al.*, 2006; Patil and Raghavarao, 2007), and treatment with different compounds (lysozyme, rivanol, Triton X-100), and extraction with acetone (Boussiba and Richmond, 1979; Tchernov *et al.*, 1999; Campanella *et al.*, 2000; Bermejo *et al.*, 2002a). It was found that live *Klebsiella pneumonia* effectively lyses *S. platensis* and extract PCs in 24 h (Zhu *et al.*, 2007).

13.4.2 Purification of phycocyanins

After the first step, consisting of cell-wall disintegration, PCs are normally precipitated using ammonium sulfate. The second step involves one or more chromatographic processes. Several chromatographic methods have been described in the literature and these involve adsorption, hydrophobic interaction, gel filtration, or ion exchange chromatography (Bermejo

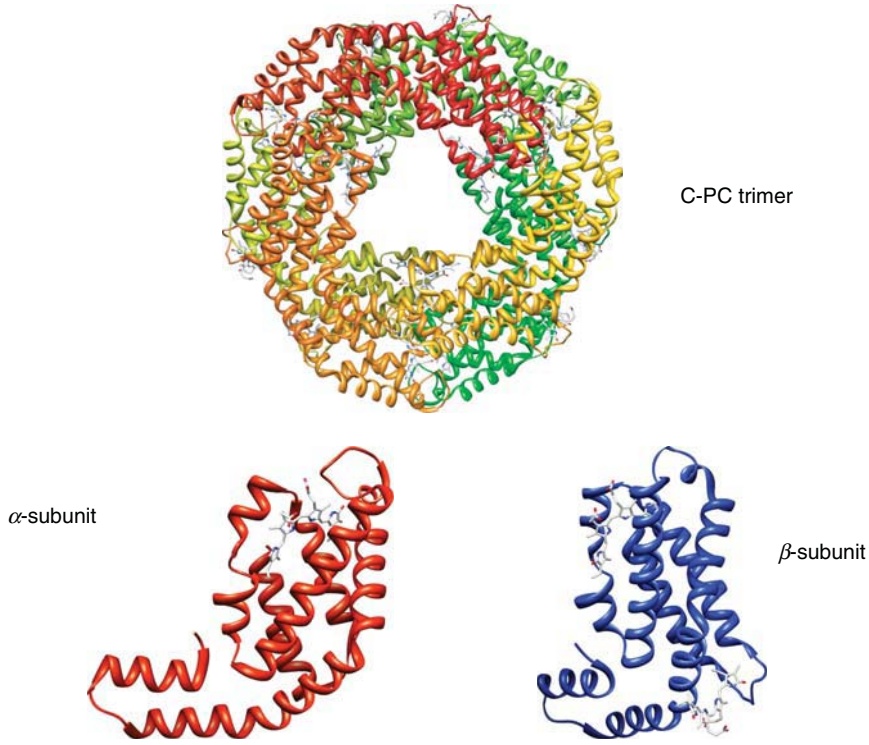

Figure 13.4 Locations and conformations or the nine phycocyanobilins in the crystal structure of the *Spirulina platensis* C-PC trimer and its subunits. See plate section for color version.

et al., 1997, 2002a; Silveira *et al.*, 2008). These methods involve a number of complex steps and are time-consuming and have a low product yield; they are therefore expensive and difficult to scale up. Hence there is a need for efficient and economic large-scale bioseparation methods that can achieve high purity as well as high yield, while maintaining the biological activity of the molecules.

To establish a competitive biotechnological process for protein purification, expanded-bed adsorption chromatography (EBAC) has been proposed. EBAC is as a single-unit operation combining separation, concentration, and capture of the target protein (Chase, 1994). The use of EBAC simplifies the clarification steps and produces a concentrate, including the partially pure product, ready for the next purification step, which is usually packed-bed chromatography (Anspach *et al.*, 1999; Clemmitt and Chase, 2000; Bermejo *et al.*, 2003b). EBAC can be used for the purification of general proteins (Anspach *et al.*, 1999) and more specifically in PCB purification. There are different studies about PC purification using EBAC at small, medium, and large scales, obtaining diverse protein quantities but always of the order of hundred milligrams (Bermejo *et al.*, 2006; Ramos *et al.*, 2010; Niu *et al.*, 2007). In fact, the use of EBAC simplifies the downstream-processing flow sheets for the recovery of a wide variety of proteins, with concomitant savings in equipment and operating costs (Bermejo *et al.*, 2006; Niu *et al.*, 2007; Ramos *et al.*, 2010, 2011).

In view of the increasing demands for relatively large amounts of PCs, a pilot-scale plant has been developed for the recovery of C-PC from *Spirulina platensis* using EBAC (Bermejo and Ramos, 2012). The EBAC technology was first optimized on a small scale, the chromatographic parameters of sample load, viscosity, and expansion degree being studied. In the next phase, the process was scaled up 100 times by increasing the column diameter, while parameters such as the settled bed height, linear flow-rate, and protein

load remained constant. The results showed a step change compared to previous results obtained for PC purification and hence this methodology could be used for industrial applications. EBAC allows food-grade PC concentrates to be obtained, ready to be loaded into conventional chromatographic columns, on which the product can be purified to obtain reactive and analytical grade PCs.

Table 13.6 lists the different procedures that have been used to purify PCs from cell extracts. Ammonium sulfate precipitation combined with a variety of chromatographic procedures has been employed to obtain PCs of food, reactive, and analytical grade (Abalde *et al.*, 1998; Minkova *et al.*, 2003; Benedetti *et al.*, 2006; Soni *et al.*, 2006; Niu *et al.*, 2007). Moreover, a two-phase extraction approach has been developed into an efficient method for PC purification, resulting in high-purity PC preparations with high yields (Rito-Palomares, Nunez, and Amador, 2001; Patil *et al.*, 2006; Patil and Raghavarao, 2007). Two-phase aqueous extraction followed by ion exchange chromatography can result in very pure C-PC (Soni, Trivedi, and Madamwar, 2008).

In all of the methodologies developed for PC purification, the purity of the PCs obtained has been confirmed by SDS-PAGE electrophoresis, spectroscopic characterization (absorbance, fluorescence, and steady state fluorescence anisotropy), and RP-HPLC.

13.5 Applications

Since its introduction in 1982, the application of PCBs in general and PCs in particular have continued to expand. This longstanding popularity rests on a number of unique properties of these proteins. The recent research and developments in PC synthesis and functionality have further expanded the potential applications of these macromolecules in the areas of biotechnology, diagnostics, food, and medicine.

13.5.1 Fluorescent probes

In the first step of purification, when phycobilisomes are extracted into aqueous buffers they disintegrate, resulting in PCBs losing excitation energy and becoming highly fluorescent. Compared to other fluorophores, these molecules have high molar extinction coefficients and fluorescence quantum yields, and large Stokes shifts (see Table 13.4). It is important to emphasize that they can form bonds to other molecules. For example, PCs conjugated to immunoglobins, protein A, and avidin have been developed into fluorescent probes. These have been widely used in histochemistry, fluorescence microscopy, flow cytometry, fluorescent-activated cell sorting, and fluorescence immunoassays (Glazer and Stryer, 1984; Glazer, 1994; Bermejo *et al.*, 2002b; Sun *et al.*, 2003; Chowdhury *et al.*, 2007; Sekar and Chandramohan, 2008). The high molar extinction coefficients are due to many chromophores per PCB complex, and this is higher for $(\alpha\beta)_6$ hexamers and $(\alpha\beta)_3$ trimers than for $\alpha\beta$ monomers. Moreover, the extinction coefficients of individual bilins decrease when hexamers disintegrate into trimers and monomers (Thoren *et al.*, 2006), while the fluorescence quantum yield decreases when PCB complexes dissociate and the chromophores gain increased conformational freedom (Glazer and Stryer, 1984; Kupka and Scheer, 2008). Thus extinction coefficients diminish and fluorescence is almost lost in denatured PCBs.

Compared to PCs, C-PC and APC are more valuable, and are normally dissolved in dilute buffer below 30 mM. At PC concentrations below 1 μM, monomers are dominant and the fluorescent yield low (Sun *et al.*, 2003; Kupka and Scheer, 2008). APC as a fluorescent probe depends on chemical cross-linking of peptides to form stable trimers (Yeh *et al.*, 1987). Moreover, C-PC trimers can be chemically stabilized by cross-linking of peptide chains (Fukui *et al.*, 2004; Sun, Wang and Qiao, 2006). Absorbance and fluorescence emission spectra of the chemically stabilized C-PC trimers are very similar to those of the native C-PC trimers, except that their extinction coefficients increase. These stabilized trimers can be used in fluorescent probes with spectral properties different from other PCBs. Stabilized C-PC and APC are combined with streptavidin and used as fluorescent probes in cytometry (Telford *et al.*, 2001).

PCs have been widely used in a variety of fluoroimmunoassays. The natural ability of PCs to participate in efficient fluorescence–resonance energy transfer (FRET) processes have also been exploited in various FRET assays with commercially available antibodies. It is also used in flow cytometry applications to study arrays of biomolecules and multimolecular complexes (Kronick and Grossman, 1983). PCs-Fab conjugates

Table 13.6 Procedures used to purify phycocyanins from cyanobacterial extracts.

Numbers and types of central unit operations	Cyanobacteria	Reference
Chromatographic methods		
1. $(NH_4)_2SO_4$ precipitation 2. Hydroxyapatite chromatography 3. Ion exchange chromatography	*Spirulina platensis*	Boussiba and Richmond (1979)
1. $(NH_4)_2SO_4$ fractionation 2. Ion exchange chromatography 3. Gel filtration	*S. platensis*	Zhang and Chen (1999)
1. Rivanol treatment 2. $(NH_4)_2SO_4$ precipitation 3. Gel filtration 4. $(NH_4)_2SO_4$ precipitation	*S. fusiformis*	Minkova *et al.* (2003)
1. Expanded bed adsorption chromatography 2. Anion exchange chromatography	*S. platensis*	Niu *et al.* (2007)
1. Hydrophobic interaction chromatography 2. Ion exchange chromatography	*Synechococcus* sp.	Abalde *et al.* (1998)
1. $(NH_4)_2SO_4$ precipitation 2. Size exclusion chromatography 3. Anion exchange chromatography	*Oscillatoria quadripunctulata*	Soni *et al.* (2006)
1. $(NH_4)_2SO_4$ precipitation 2. hydroxyapatite (electrostatic interaction) chromatography	*Aphanizomenon flos-aquae*	Benedetti *et al.* (2006)
1. $(NH_4)_2SO_4$ fractionation 2. Hydrophobic interaction chromatography	*Phormidium fragile*	Soni, Trivedi, and Madamwar (2008)
1. Expanded bed adsorption chromatography 2. Ion exchange chromatography (large scale)	*Anabaena marina*	Ramos *et al.* (2010)
1. Expanded bed adsorption chromatography 2. Ion exchange chromatography (large scale)	*S. aquatilis*	Ramos *et al.* (2011)
1. Expanded bed adsorption chromatography 2. Ion exchange chromatography (pilot scale)	*S. platensis*	Bermejo *et al.* (2012)
Two-phase aqueous extraction methods		
1. Two-phase aqueous extraction 2. Ultrafiltration 3. $(NH_4)_2SO_4$ precipitation	*S. maxima*	Rito-Palomares, Nunez and Amador (2001)
1. Repeated two-phase aqueous extraction 2. Ultrafiltration	*S. platensis*	Patil and Raghavarao (2007)
1. Chitosan adsorption 2. Two-phase aqueous extraction	*S. platensis*	Patil *et al.* (2006)
Combined two-phase aqueous extraction and chromatographic methods		
1. Chitosan adsorption, 2. Two-phase aqueous extraction, 3. Ion exchange chromatography	*S. platensis*	Patil *et al.* (2006)

have been used in single particle fluorescence imaging applications for observing cell receptor movements and associations at high spatial resolution on the surface of living cells (Triantafilou, Triantafilou, and Wilson, 2000). PCs have been the focus of several single-molecule spectroscopy studies and have been used in high-speed high-throughput single-molecule imaging techniques for identification of free solution molecules, a technique with potential for use in high-speed detection of specific disease markers (Loos *et al*., 2004).

In vivo fluorescence from PCs can be used for online monitoring of growth in cyanobacterial cultures (Sode *et al*., 1991), detection of toxic cyanobacteria in drinking water (Izydorczyk *et al*., 2005), and remote sensing of cyanobacteria in natural waters (Simis, Peters, and Gons, 2005). The fluorescent properties of PCs have been utilized to label nucleic acid to detect hybridization by means of steady-state fluorescence anisotropy in homogeneous aqueous solution, an easy method to obtain PC-DNA conjugates (Bermejo *et al*., 2002b).

Table 13.7 Health potential of phycocyanins; suggested physiological mechanisms responsible for the effects and experimental system.

Pharmaceutical potential and physiological mechanism of phycocyanin	**Experimental system**	**Reference**
Anti-inflammatory, prevents glucose oxidase-induced edema	Mouse, *in vivo*	Romay *et al*. (1998)
Anti-inflammatory, reduces acetic acid-induced colitis, scavenging of oxygen radicals	Rat, *in vivo*	González *et al*. (1999)
Prevents cholesterol-induced artherosclerosis, improved antioxidant effect in plasma lipoproteins	Hamster, *in vivo*	Riss *et al*. (2007)
Prevents kainic acid-induced neural damage, scavenging of oxygen radicals	Rat, *in vivo*	Rimbau *et al*. (1999)
Prevents oxalic acid-induced kidney stone formation, prevents lipid peroxidation	Rat, *in vivo*	Farooq *et al*. (2004)
Prevents thioacetamide-induced hepatic encephalopathy, prevents lipid peroxidation	Rat, *in vivo*	Sathyasaikumar *et al*. (2007)
Reduces cardiotoxicity of the drug doxorubicin, scavenging of oxygen radicals	Rat cardiomyocetes	Khan *et al*. (2006)
Reduces cholesterol	Human, rat	Henrikson (1994)
Reduces mouth cancer	Hamster	Henrikson (1994)
Proliferation of lactobacillus	Rat	Henrikson (1994)
Reduction of renal poisoning by heavy metals and drugs	Rat	Henrikson (1994)
Increased bioavailability of iron	Rat	Henrikson (1994)
Correction of iron deficiency anemia	Human, rat	Henrikson (1994)
Anti-inflammatory, represses nitric oxide synthase expression and reduce nitrite synthesis	Macrophage cell line	Cherng *et al*. (2007)
Anti-platelet aggregation, predominantly due to inhibition of cyclooxygenase	Rabbit plasma	Chiu *et al*. (2006)
Anti-cancerogenic, induces apoptosis in human hepatocellular-carcinoma cells	Hepatocellular carcinoma cells	Roy *et al*. (2007)
Anti-cancerogenic, inhibits proliferation of human myeloid leukemia cells	Myelogenous leukemia blast cells	Liu *et al*. (2000)
Anti-cancerogenic, induces apoptosis in human myeloid leukemia cells	Myelogenous leukemia blast cells	Subhashini *et al*. (2004)

Another important application is the development of new bio-nanomaterials and nanostructures using PCs. The use of plasmonic metal nanostructures in the form of silver island films to enhance the fluorescence emission of different PCs has been reported (Chowdhury *et al.*, 2007). A major challenge in this area is to build multifunctionality into a nanostructure, especially the incorporation of fluorescence functionality in magnetic nanoparticles, which would have considerable value in areas like the biomedical field. Bi-functional nanoparticles have been built using ferritin linked with PCs; the coexistence of these two properties (magnetism and fluorescence) assists *in situ* detection and monitoring by fluorescence imaging of the nanoparticles' movement under an external magnetic field. This is of enormous interest both from the therapeutic and diagnostic points of view (Bermejo *et al.*, 2011).

13.5.2 Health compounds

Purified PCs have immense health potential as nutraceutical and pharmaceutical compounds. The antioxidant and radical-scavenging properties of C-PC are well documented (Romay *et al.*, 1998; Bhat and Madyastha, 2000; Benedetti *et al.*, 2004; Bermejo, Pinero, and Villar, 2008; Soni, Trivedi and Madamwar, 2008). C-PC is bleached during scavenging of peroxyl radicals (Hirata *et al.*, 2000; Bhat and Madyastha, 2000). The antioxidative activity of free phycocyanobilin is comparable to phycocyanobilin bound in C-PC (Lissi, Pizarro and Ramay, 2000), which is increased by denaturing or by trypsin digestion of C-PC (Zhou *et al.*, 2005). The apo-protein and recombinant apo-APC with no bilins attached also have antioxidative properties (Ge *et al.*, 2006).

Enhanced radical-scavenging activities have been found in selenium-enriched C-PC. It is to be noted that Se-PC can be obtained from. *S. platensis* grown in an Se-enriched medium (Huang *et al.*, 2007). It has also been reported that C-PC can inhibit the activity of the enzyme NADH oxidase (as well as others), affects gene regulation in mammalian cell lines (Cherng *et al.*, 2007) and hamsters (Riss *et al.*, 2007), inhibits cell proliferation (Liu *et al.*, 2000), and induces apoptosis in carcinogenic cell lines (Subhashini *et al.*, 2004; Roy *et al.*, 2007). These effects have renewed interest in PCs as nutraceuticals or pharmaceuticals with anticarcinogenic and other possible health effects (Table 13.7). Besides their anticarcinogenic (Remirez *et al.*, 1999; Rimbau *et al.*, 1999), antioxidative, and anti-inflammatory activities (Bhat and Madyastha, 2000; Benedetti *et al.*, 2006), PCs have been useful in the treatment of Alzheimer's and Parkinson's disease (Rimbau *et al.*, 2001).

13.5.3 Food additives

It is important to highlight that PCs are marketed as food and cosmetic colorants in Japan, where algal cultivation is a well-developed industry. Some combinations of PCs and other PCBs have already been patented for the coloring of fermented milk products such as yogurt (Dainipon patent, 1979; Prasanna *et al.*, 2007), although they have not yet been approved in the EU. Limited consumption of blue foods and the lack of methods to obtain the macromolecules cheaply and on a large-scale have probably limited the industry's interest in PCs for food coloring. Studies have addressed the functionality of C-PC in foods with regards to color stability (Jespersen *et al.*, 2005) and rheological properties (Batista *et al.*, 2006).

It is to be emphasized that the largest route for human intake of PCs is in non-purified forms via extracts from cyanobacteria (Spolaore *et al.*, 2006). However, such extracts may also contain a range of other biologically active compounds (Jensen, Ginseberg, and Drapeau, 2001; Singh, Kate, and Banerjee, 2005). Therefore, it is difficult to attribute health effects from direct consumption of whole cyanobacteria to the PC content.

The search for natural colorants is an important area of investigation, because there is a general tendency to use natural rather than synthetic dyes in foods and cosmetics. PCs are good candidates, and have been utilized as natural colorants in milkshakes, yogurts, ice-creams, and isotonic beverages.

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Chapter 14

Cyanobacterial polyhydroxyalkanoates: an alternative source for plastics

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14.1 Introduction

Non-renewable fossil fuels are the primary resources for the energy and materials required for the maintenance of modern society. However, due to the heavy burden these place on current resources, their use is not sustainable. Use of mineral oil-based plastics (traditional plastics or petroplastics), which constitute one of the essential commodities in modern life, has increased at an annual growth rate of 9% since 1950 (Plastics Europe, 2009). A recent estimate showed that global plastics production reached around 280 million tonnes in 2011, up from 265 million tonnes in 2010, a rise of 3.7% (Plastics Europe, 2012).

Almost every aspect of daily life involves plastics. The widespread use and popularity of plastic materials lie in their mechanical properties, high thermal and electrical insulation properties, their lightness, strength, and durability, corrosion-resistance, and their low cost. Over the years there has been a rapid development in the number, type, and quality of plastic, from the first thermoplastic "celluloid" to superior modern materials such as epoxies, polycarbonates, teflons, silicones, polysulfones, nylons and so on, which have been successfully used as substitutes for many substances such as glass and ceramics, stone, horn and bone, leather, paper, metal, and so on (Lee *et al.*, 1991; Cain, 1992; Lee, 1996).

Despite their many uses and benefits, plastics have one major drawback. Upon disposal, they are not readily degraded in the environment, thereby causing a substantial burden on solid waste facilities. Plastic wastes constitute approximately 10% by weight of municipal wastes (Barnes *et al.*, 2009). Accumulation

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of plastic wastes in water bodies, landfills, and other terrestrial compartments negatively affects wildlife as well as human health. In the oceans, marine organisms are dying due to starvation as their stomachs become packed with indigestible plastic materials that have been swallowed. They may become trapped or strangled and/or drown because of the plastic wastes. The majority of plastics remain in the environment for many years, as they are extremely resistant to microbial attack as a result of their unusual molecular bonds, the presence of a large number of aromatic rings, and their high molecular mass (Alexander, 1981). Plastics are mainly of single or short-term use, after which they are dumped in landfills. However, finding enough space for landfills is a growing problem.

In developed countries (e.g., Western Europe, the United States), resource reduction, recycling, and incineration are being practiced as substitutes for land disposal (Bohlmann and Toki, 2004). However, these methods too, lead to environmental problems. Incineration is common in countries such as Japan, where land is scarce, but it is expensive and releases toxic pollutants such as dioxins, polycyclic aromatic hydrocarbons, furans, and hydrogen cyanide. (Johnstone, 1990; Atlas, 1993). In particular, burning polystyrene foam products releases chlorofluorocarbons and hydrochlorofluorocarbons, which are ozone destroying and greenhouse gases (Chiras, 1994). Recycling is good, but very tedious and not always cost-effective. Sorting of the wide varieties of discarded plastic materials is a time-consuming process. Moreover, the applications of recycled materials are limited due to the presence of additives such as pigments, coatings, and fillers. Thus increasing awareness of the negative environmental impacts of the petroplastic wastes has driven a movement to explore novel raw materials under the "green agenda." This has led to efforts to produce bioplastics (biodegradable polymers).

The main challenge in the production of bioplastics is to maintain the physical and chemical nature of the petroleum-based plastics, while adding the advantage of biodegradability (Lee, 1996; Amass, Amass, and Tighe, 1998). Biodegradability is defined as degradation of polymers by environmental factors (e.g., light, heat, chemical conditions) and/or by biological activity. With the possible exception of coral reefs, the biological world readily degrades what it builds. Thus biodegradable materials are a creation of life itself (Braunegg, Gilles, and Klaus, 1998).

Degradable plastics can be subdivided into three broad types: photodegradable, semi-biodegradable, and completely biodegradable (Reddy *et al.*, 2003). Photodegradable plastics have light-sensitive groups incorporated directly into the backbone of the polymer as additives. These plastics can be degraded by ultraviolet radiation (Gugumus, 1990; Pospíšil and Nešpůrek, 1997). However, the absence of sunlight in landfills prevents their degradation. The second type, the starch-linked plastics, commonly known as semi-biodegradable plastics, are degraded by soil microbes on disposal, but only the starch units: the polyethylene fragments are left undegraded in the soil (Johnstone, 1990).

The third type of bioplastic is completely biodegradable by microorganisms. Polycaprolactone and polybutylene succinate are petroleum-based plastics, but are degraded by microorganisms. Polyhydroxyalkanoates (PHAs), polylactides, polysaccharides, and/or blends of them are based on renewable resources, and are completely biodegradable (Tokiwa *et al.*, 2009). Amongst microbial products, PHAs have a great potential as green substitutes for petroleum-derived polymers. Other bioplastics such as polyglycolic acid and polylactic acid are chemically synthesized, but they lack structural variability and wide-ranging material properties (Keshavarz and Roy, 2010).

14.2 Polyhydroxyalkanoates and their types

Polyhydroxyalkanoates (PHAs) are polyesters of hydroxyl acids synthesized mainly by prokaryotes and some plants and animal cells. However, only prokaryotes are capable of accumulating high molecular weight polymers as a carbon reserve and an electron sink. PHAs are accumulated intracellularly in the form of amorphous inclusions, with negligible osmotic activity, at times of oversupply of carbon sources and limited essential nutrients in the growth medium (Anderson and Dawes, 1990). PHAs are of ecological interest as they undergo rapid degradation under aerobic as well as anaerobic conditions (Tokiwa

et al., 1992), and even under thermophilic conditions (Calabia and Tokiwa, 2004). A large number of microorganisms have been found that use their own secreted enzymes, PHA depolymerases, to degrade PHAs into monomers and oligomers at a high rate (Jendrossek, 2001). These are later metabolized by the microbes, producing water and carbon dioxide as end products under aerobic conditions, and carbon dioxide and methane under anaerobic conditions. However, it is not only the biodegradability of these polymers that makes PHAs fascinating, but also the possibility of their synthesis from renewable sources in a sustainable closed cycle (Braunegg, Gilles, and Klaus, 1998). Other important features of PHAs are that they are elastomeric, insoluble in water, enantiomerically pure, non-toxic, biocompatible, piezoelectric, and have high degrees of polymerization with molecular weights of several million Daltons (Müller and Seebach, 1993; Hocking and Marchessault, 1994).

In most PHA polymers, the β-position carbon atom is always in the *R* configuration, varying from methyl to tridecyl. It can either be saturated or unsaturated, branched, epoxidized, aromatic, or halogenated (Abe *et al.*, 1990; Kim, Lenz, and Fuller, 1991, 1992; Choi and Yoon, 1994; Song and Yoon, 1996). These variations in the length and composition of the side chains and the possibility of altering their reactive substituents are the basis for the diversity of the PHA polymer family and its huge range of prospective applications (Madison and Huisman, 1999).

With more than 150 different types of monomer composition exhibiting different properties and functionalities, PHAs comprise the largest group among biopolymers (Hazer and Steinbüchel, 2007). Depending upon the size of their comprising monomers, PHAs can be subdivided into three classes. PHAs containing 3–5 carbon monomers are classified as short chain length (SCL-PHA); those with 6–14, and more than 14 carbon monomers are classified as medium chain length (MCL-PHA) and long chain length (LCL-PHA), respectively. SCL-PHAs are stereo-regular polymers with all the asymmetric carbon atoms in the *R*-stereochemical configuration. As a consequence, the polymer is isotactic, highly crystalline, and relatively stiff. Its mechanical properties, such as Young's modulus and the tensile strength, and its thermal properties such as the glass-to-rubber transition temperature (T_g) and the melting temperature (T_m) are quite similar to those for polypropylene. The MCL-PHAs are regarded as elastomers, characterized by low T_m and high values of elongation-to-break (Lee, 1996). PHAs made of these monomers are sticky and can be modified to make rubbers. Until 2008, there were no reports of LCL-PHAs. The first bacterium found to synthesize a novel short-chain-length-long-chain-length co-polymer with 3-hydroxybutyric acid (3HB), 3-hydroxyvaleric acid (3HV), 3-hydroxyhexadecanoic acid (3HHD), and 3-hydroxyoctadecanoic acid (3HOD) as its constituents was a sludge isolate, identified as *Pseudomonas aeruginosa* MTCC 7925 (Singh and Mallick, 2008; 2009a,b). This novel copolymer exhibited material properties comparable with those of polypropylene and low-density polyethylene.

Poly-β-hydroxybutyrate (PHB), a homopolymer of 3-hydroxybutyric acid is the most common and extensively studied SCL-PHA. However, the homopolymer PHB is quite rigid and brittle, which limits its use. Copolymers of PHAs can be obtained by polymerization between more than one kind of monomer, for example 3HB, 3HV, 3-hydroxyhexanoate, or 4-hydroxybutyrate monomers. SCL-PHAs containing mainly 3HB units or MCL-PHAs containing 3-hydroxyoctanoate and 3-hydroxydecanoate are the major monomers synthesized by the majority of microbes (Anderson and Dawes, 1990; Steinbüchel, 1991; Steinbüchel and Schlegel, 1991; Lee, 1996).

14.3 Applications of PHA polymers

Properties such as biodegradability, biocompatibility, and hydrophobicity give PHAs a wide applicability, for example in medical implants, drug delivery carriers, printing and photographic materials, in addition to their more conventional use as packaging and disposable materials. Recently, PHA polymers have also been considered as a new type of feedstock for biofuel (Zhang *et al.*, 2009). PHA-related proteins and genes have been used to regulate metabolism and improve the toughness of industrial microorganisms (Liu *et al.*, 2007). In addition, PHAs might have uses in specific drug targeting (Yao *et al.*, 2008) and protein

purification (Rehm, 2007; Wang *et al.*, 2008). The list of PHA applications is expanding rapidly (Table 14.1).

Although the history of the commercialization of PHAs dates back to 1959, most of the applications of PHA are still under research. W.R. Grace and Company has produced the first PHBs for commercial applications (Baptist, 1965). However, due to low production levels and unsuitable purification methods, the company later stopped their manufacture. In 1970, Imperial Chemical Industries commercialized poly-3-hydroxybutyrate-*co*-3-hydroxyvalerate under the trade name Biopol™, the rights to which were later sold to Monsanto and then to Metabolix. Subsequently, many companies, alone or in collaboration with a research institute, have commercialized PHAs (Table 14.2). Procter and Gamble, in collaboration with Kaneka Corporation, Tsinga University, and the Riken Institute, have developed a range of products under the trade name Nodax™. Other companies currently engaged in the production of bioplastics are BioMer (Germany) and Mitsubishi Gas Chemical (Japan), using the trade names of Biomer® and Biogreen®, respectively. In 2006, the cost of PHBs was very high (in the range of €10/kg; Kosior, Braganca, and Fowler, 2006). The latest market price of a PHB under the trade name of Mirel™ is about €1.50/kg (Chanprateep, 2010). By 2020, a mammoth scale rise in the bioplastics market is forecast (Chen, 2009). with many new applications in the automotive, textile and agricultural sectors coming on stream.

14.4 Production of PHA polymers

14.4.1 Bacterial fermentation

More than 300 species of bacteria (e.g., *Cupriavidus necator*, *Pseudomonas* sp., *Bacillus* sp., *Methylobacterium* sp.) have the ability to biosynthesise PHAs. Using fermentation technology, the maximum PHB that can be accumulated by heterotrophic bacteria amounts to up to 80% of the dry cell weight (dcw; Reddy *et al.*, 2003). To have a clear picture of bacterial PHA production, readers are referred to Lafferty, Korsatko, and Korsatko (1988); Anderson and Dawes (1990); Doi (1990); Fuller and Lenz (1990); Lee (1996); Sasikala (1996); Steinbüchel (1996); Braunegg, Gilles, and Klaus, (1998); Lee, Choi, and Wong (1999); Sudesh, Abe, and Doi (2000); Ishizaki, Tanaka, and Taga (2001); Serafim *et al.* (2001), Reddy *et al.* (2003), Khanna and Srivastava (2005), Lenz and Marchessault (2005), Valappil *et al.* (2007), Suriyamongkol *et al.* (2007), Chen (2009), Keshavarz and Roy (2010) and Sudesh *et al.* (2011). Despite being under investigation for such a long time, large-scale PHA production is still to be optimized. The major drawback associated with commercial-scale PHA-based plastic production is the high production cost, particularly the cost of the substrates and the continuous oxygen supply required during bacterial fermentation. This difficulty has impelled the use of photoautotrophic systems for PHA-based plastic production.

14.4.2 Photoautotrophic production

14.4.2.1 Transgenic plants as production hosts

PHA synthesis has been reported in some transgenic plants (e.g., *Arabidopsis*, cotton, alfalfa, flax, sugarcane, tobacco, rapeseed), through engineering of novel pathways in the cytoplasm, plastids, or in the peroxisomes (Nawrath, Poirier, and Somerville, 1994; Slater *et al.*, 1999; Mitsky *et al.*, 2000; Romano *et al.*, 2003, 2005; Somleva *et al.*, 2008; Murthy *et al.*, 2010; Dalton *et al.*, 2011). Plants could be potential PHA-production systems, because β-ketothiolase, which controls the first step of PHB biosynthesis, is already present in their cytoplasm. Engineering *Arabidopsis thaliana* with the other two enzymes of the PHB biosynthetic pathway, namely acetoacetyl-CoA reductase and PHA synthase, in the cytoplasm, leads to accumulations of PHB of up to 0.1% of plant dry weight (Poirier *et al.*, 1992). PHB synthesis has also been reported in transgenic tobacco (0.01% of dry weight), cotton (0.3%), and oilseed rape (0.1%) (John and Keller, 1996; Nakashita *et al.*, 1999; Poirier and Gruys, 2001). As this shows, the accumulation potential is very low and all the plants showed severe retardation in growth and abridged seed production. High production levels are however, attainable, by targeting the PHB pathway to plastids. In a study, three *Wautersia eutropha* (now known as *C. necator*)

Table 14.1 Potential applications of PHA polymers.

Application	Example	Property	Reference
Disposables	Razors, tray for foods, utensils, etc.	Biodegradability, good mechanical properties	Lauzier (1992)
Hygiene products	Diapers, feminine hygiene products	Moisture resistance, good water barrier, biodegradability	Weiner (1997)
Healthy food additives	PHA oligomers can be used as food supplements for obtaining ketone bodies	Source of 3-hydroxyacids	Martin *et al.* (1999)
Bulk chemicals	Heat sensitive adhesives, latex, smart gels, PHA matrices can be used to remove facial oils	Stereoregularity, chiral properties	Lee, Choi, and Wong (1999)
Textile industry	PHA can be processed into fibers	Moisture resistance, high tensile strength	Perepelkin (2005)
Fine chemical industry	PHA monomers are all chiral R-forms, and can be used as chiral starting materials for the synthesis of antibiotics	Stereoregularity, chiral properties	Chen and Wu (2005a)
Medical	PHA used as sutures, repair devices, repair patches, slings, cardiovascular patches, orthopedic pins, adhesion barriers, stents, guided tissue repair/regeneration devices, articular cartilage repair devices, nerve guides, tendon repair devices, bone marrow scaffolds, artificial oesophagus, dressings, etc.	Biocompatibility, biodegradability	Chen and Wu (2005b)
Industrial microbiology	The PHA synthesis operon can be used as a metabolic regulator or resistance enhancer to improve the performance of industrial microbial strains	–	Müller, Bley, and Babel (1999); Zhang, Hao, and Chen (2006)
Packaging industry	All packaging materials that are used for a short period of time, including food utensils, films, daily consumables, electronic appliances, etc.	Biodegradability, good mechanical properties, moisture resistance, low oxygen permeability	Philip, Keshavarz, and Roy (2007)
Miscellaneous	Autoseparative air filters, biodegradable goods	Biodegradability, good mechanical properties	Philip, Keshavarz, and Roy (2007)
Agriculture	Controlled release of pesticides, plant growth regulators, herbicides, fertilizers	Biodegradability, retarding properties	Kyrikou and Briassoulis (2007)
Protein purification	PHA granule binding proteins phasin or PhaP are used to purify recombinant proteins	Hydrophobic	Rehm (2007); Wang *et al.* (2008)
Block copolymerization	PHA can be changed into PHA diols for block copolymerization with other polymers	Chiral properties	Liu, Goh, and Li (2008)
Specific drug delivery	Co-expression of PhaP and specific ligands can help to achieve specific targeting to diseased tissues	Biodegradability, biocompatibility	Yao *et al.* (2008)
Medical implant biomaterials	PHA was successfully used as an osteosynthetic material for stimulating bone growth and effectively repairing damaged nerves	Biocompatibility, piezoelectric properties	Wang *et al.* (2008); Bian *et al.* (2009)

(*continued overleaf*)

Table 14.1 (*continued*)

Application	Example	Property	Reference
Biofuels or fuel additives	PHA can be hydrolyzed to form hydroxyalkanoate methyl esters that are combustible	–	Zhang *et al*. (2009)
Printing and Photographic industry	PHA are polyesters that can be easily stained	Chiral purity	Li *et al*. (2009)
Plastic processing	PHA can be used as processing aids for plastic processing	Good mechanical properties	Chen (2009)

Compiled from Vincenzini and De Philippis, 1999; Philip, Keshavarz, and Roy, 2007; and Chen, 2009.

Table 14.2 Worldwide PHA producing and research oriented companies.

Company	Type of PHA	Period	Application
ICI, UK	PHBV	1980s to 1990s	Packaging
Chemie Linz, Austria	PHB	1980s	Packaging and drug delivery
BASF, Germany	PHB, PHBV	1980s to 2005	Blending with Ecoflex
Monsanto, USA	Several PHAs	1980s to present	Packaging
P&G, USA	Several PHAs	1980s to 2005	Packaging
btF, Austria	PHB	1990s	Packaging & drug delivery
Biomers, Germany	PHB	1990s to present	Packaging & drug delivery
Tepha, USA	Several PHAs	1990s to present	Medical bio-implants
Metabolix, USA	PHB, PHBV	1990s	Raw materials
Kaneka, Japan (with P&G)	Several PHAs	1990s to present	Packaging
Mitsubishi, Japan	PHB	1990s	Packaging
Biocycles, Brazil	PHB	1990s to present	Raw materials
Zhejiang Tian An, China	PHBV	1990s to present	Raw materials
Jiangmen Biotech Ctr, China	PHBHHx	1990s	Raw materials
Tianjin Northern Food, China	PHB	1990s	Raw materials
Shantou Lianyi Biotech, China	Several PHAs	1990s to 2005	Packaging and medical
Jiang Su Nan Tian, China	PHB	1990s to present	Raw materials
Shenzhen O'Bioer, China	Several PHAs	2004 to present	Unclear
Tianjin Green Bio-Science, China	P3HB4HB	2004 to present	Raw materials and packaging
ADM, USA (with Metabolix)	Several PHAs	2005 to present	Raw materials
Shandong Lukang, China	Several PHAs	2005 to present	Raw materials & medical
Meredian, USA	Several PHAs	2007 to present	Raw materials
Bio-On, Italy	PHA (unclear)	2008 to present	Raw materials
Yikeman, Shandong, China	PHA (unclear)	2008 to present	Raw materials

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genes modified for plastid targeting were expressed in *A. thaliana* using separate T-DNA vectors. This approach increased the amount of PHB to a maximum of 14% of dry weight in leaves without any adverse effect on plant growth (except leaf chlorosis). However, these transgenes were not stable in the progeny (Nawrath, Poirier, and Somerville, 1994). However, production of PHBs up to levels of 30–40% of shoot dry weight have since been achieved by engineering the same genes in a single T-DNA vector (Bohmert *et al.*, 2000). Growth of the transgenic plants was however, severely reduced, with no seed production. To circumvent this problem, Kourtz *et al.* (2007) transformed *Arabidopsis* with a multigene construct with a gene switch, the accumulation of PHB was only up to 7 and 14% of dry weight, respectively, in young and old leaves. Thus, efforts to optimize PHA production in plants has led to a few success stories. However, low levels of expression, long growth periods, and difficulties in isolating the PHA from cellular components are among the disadvantages associated with plant-based PHA production.

14.4.2.2 Cyanobacteria as production hosts

Cyanobacteria have the ability to accumulate PHAs. Carr (1966) used chemical methods to demonstrate PHB accumulation in *Chlorogloea fritschii*, while Jensen and Sicko (1973) used electron microscopy to show PHB granules in the same cyanobacterium. Since then, PHB has been detected in many species of cyanobacteria, for example *Gloeocapsa* sp. (Rippka *et al.*, 1971), *Spirulina platensis* (Campbell, Stevens, and Bankwill, 1982), *Aphanothece* sp. (Capon *et al.*, 1983), *Oscillatoria limosa* (Stal, Heyer, and Jacobs, 1990), *Anabaena cylindrica* (Lama *et al.*, 1996), *Synechococcus* sp. (Miyake, Erata, and Asada, 1996), *Synechocystis* sp. (Sudesh, Taguchi, and Doi, 2002) and *Nostoc muscorum* (Sharma and Mallick, 2005a). Generally, estimates of the PHB content in cyanobacteria grown under photoautotrophic conditions are low (<10% of dcw, Table 14.3). However, Nishioka *et al.* (2001) reported PHB accumulations of up to 55% dcw under phosphate-limited conditions in *Synechococcus* sp. MA19, an isolate from volcanic rocks in Japan. In another unicellular cyanobacterium, *Synechocystis* sp. PCC 6803, grown under mixotrophy with low phosphate and gas-exchange-limited conditions, accumulation was up to 38% (Panda and Mallick, 2007). Sharma *et al.* (2007) reported an accumulation of up to 46% in *Nostoc muscorum* subjected to chemoheterotrophy under phosphate limitation. Samantaray and Mallick (2012) reported a maximal accumulation of 85% PHB in *Aulosira fertilissima* under optimized conditions. Interestingly, Akiyama *et al.* (2011) demonstrated antibiotic-free PHA production (52% dcw) by the recombinant *Synechococcus* sp. PCC7002 (ATCC 27264) under nitrogen-limited conditions, where the main component of the polymer was PHB (>98%). Table 14.4 shows PHA accumulations in different cyanobacteria under mixotrophic growth conditions. In *Nostoc muscorum*, P(3HB-*co*-3HV) copolymer synthesis was detected under propionate as well as valerate-supplemented conditions (Mallick *et al.*, 2007; Bhati and Mallick, 2012). All these results demonstrate that cyanobacteria are a promising source for PHA production.

14.5 Cyanobacterial PHA production utilizing wastes

The overall economics of PHA production are affected by various factors: the cost of raw materials, PHA productivity, and the downstream processes. When the process is scaled up, the raw materials (mainly carbon sources) contribute to a major fraction of the overall production cost (Choi and Lee, 1997). Thus the economic feasibility of large-scale PHA production is essentially dependent on identifying biotechnological processes utilizing inexpensive raw materials. In this regard, use of waste discharges as media could provide an advantage, with a reduction in disposal or recycling costs, and simultaneous production of a value-added product.

Cyanobacteria have long been explored as bioremediation agents. They are highly effective accumulators and/ or degraders of pesticides, crude oil, naphthalene, phenanthrene, phenol, heavy metals, catechol, and xenobiotics (Megharaj, Venkateswarlu, and Rao, 1987). Free as well as immobilized forms can be used for this purpose. Cyanobacteria as a bioremediation agent have certain advantages over other microorganisms. Their photoautotrophic nature enables them to

Table 14.3 Reports on detection of PHAs in cyanobacteria under photoautotrophic growth conditions.

Cyanobacterial species	Culture condition	Type of polymer	Polymer content (% dcw)	Reference
Gloeocapsa PCC 6501	Photoautotrophy	PHB	P	Rippka *et al*. (1971)
Chlorogloea fritschii Mitra	Photoautotrophy	PHB	P	Jensen and Sicko (1973)
Nostoc sp. (13 strains)	Photoautotrophy	PHB	P	Jensen (1980)
Microcystis aeruginosa	Photoautotrophy	PHB	P	Jensen and Baxter (1981)
Spirulina platensis	Photoautotrophy	PHB	6	Campbell, Stevens, and Bankwill (1982)
Anacystis cyanea	Photoautotrophy	PHB	P	Sicko-Goad (1982)
Oscillatoria limosa 23	Photoautotrophy	PHV	P	Stal, Heyer, and Jacobs (1990)
Spirulina subsalsa 85	Photoautotrophy	PHB	P	
Spirulina jenneri NK1	Photoautotrophy	PHB	P	Vincenzini *et al*. (1990)
Spirulina laxissima MG5	Photoautotrophy	PHB	P	Vincenzini *et al*. (1990)
Gloeothece sp. PCC 6909	Photoautotrophy	PHB	6.4	Stal (1992)
Microcystis aeruginosa (2 strains)	Photoautotrophy	PHB	P	
Trichodesmium thiebautii	Photoautotrophy	PHB	P	Siddiqui, Carpenter, and Bergman (1992)
Anabaena cylindrica 10C	Photoautotrophy nitrogen-starved	PHB	0.2	Lama *et al*. (1996)
Synechococcus sp. MA19	Photoautotrophy nitrogen-starved	PHB	27	Miyake, Erata, and Asada (1996)
Synechococcus sp. MA19	Photoautotrophy Phosphate-limited	PHB	55	Nishioka *et al*. (2001)
Nostoc muscorum	Photoautotrophy	PHB	8.5	Sharma and Mallick (2005a)
Synechocystis sp. PCC 6803	Photoautotrophy	PHB	4.5	Panda *et al*. (2006)
Nostoc muscorum	Photoautotrophy	PHB	8.5	Bhati *et al*. (2010)
Scytonema sp.	Photoautotrophy	PHB	7.4	
Spirulina subsalsa	NaCl	PHB	7.5	Shrivastav, Mishra, and Mishra (2010)
Synechococcus sp. PCC 7002 (ATCC 27264) recombinant	Photoautotrophy Phosphate-limited	Mainly PHB	52	Akiyama *et al*. (2011)
Nostoc muscorum	Photoautotrophy (aeration + CO_2)	PHB	21.5	Haase, Huchzermeyer, and Rath (2012)

P: detected, not quantified.

be true producers. In addition, the unique ability of some of them to fix atmospheric nitrogen makes their growth and maintenance inexpensive (Castenholz and Waterbury, 1989). Forms such as *Spirulina platensis* (Gantar, Obreht, and Dalmacija, 1991; Cheunbarn and Peerapornpisal, 2010), *Anabaena doliolum* (Rai and Mallick, 1992), *Spirulina maxima* (Olguin *et al*., 1994), *Synechococcus* sp. PCC 7942 (Qiang, Westerhoff, and Vermaas, 2000), *Phormidium subfuscum*, *Oscillatoria* sp., *Phormidium tenue* and *Schizothrix calcicola* (Chevalier *et al*., 2000), and *Romeria* sp. (Burford, 2005) are highly efficient in removing nutrients such as ammonia, nitrate, phosphate, and urea. Moreover, a tropical cyanobacterium, *Phormidium bohneri* Schmidle, exhibits a self-aggregation property, which enables it to autoflocculate, enhancing its applicability

Table 14.4 Reports on accumulation of PHAs in cyanobacteria under mixotrophic growth conditions.

Cyanobacterial species	Culture condition	Type of polymer	Polymer content (% dcw)	Reference
Chlorogloea fritschii	Acetate	PHB	10	Carr (1966)
Anabaena cylindrica	Acetate	PHB	P	Carr and Bradley (1973)
Aphanocapsa PCC 6308	Acetate	PHB	P	Allen (1984)
Spirulina maxima (3 strains)	Acetate	PHB	3.1	Vincenzini *et al*. (1990) ; De Philippis, Sili, and Vincenzini, (1992)
Spirulina platensis (4 strains)	Acetate	PHB	2.9	Vincenzini *et al*. (1990) ; De Philippis, Sili, and Vincenzini, (1992)
Oscillatoria limosa	Acetate	PHV	6	Stal (1992)
Anabaena cylindrica 10C	Acetate + propionate	P(3HB-*co*-3HV)	2	Lama *et al*. (1996)
Synechocystis sp. PCC 6803 wild type	Acetate	PHB	7	Sudesh, Taguchi and Doi (2002)
Synechocystis sp. PCC 6803 recombinant	Acetate	PHB	11	
Synechocystis sp. PCC 6803	Acetate + glucose + P-limitation	PHB	29	Panda *et al*. (2006)
	Chemoheterotrophy (acetate)	PHB	22	Panda and Mallick (2007)
	Acetate +fructose + gas exchange under phosphate limitation	PHB	38	
	Optimization by RSM	PHB	43	Panda (2008)
Nostoc muscorum	Chemoheterotrophy (acetate)	PHB	43	Sharma and Mallick (2005b)
	Optimization by RSM	PHB	46	Sharma *et al*. (2007)
	Optimization by RSM	P(3HB-*co*-3HV)	31	Mallick *et al*. (2007)
Synechocystis sp. UNIWG	Mixotrophy (acetate) + nitrogen-starved + CO_2	PHB	14	Toh *et al*. (2008)
Spirulina platensis UMACC			10	
Aulosira fertilissima	Acetate + citrate + P-limitation	PHB	85	Samantaray and Mallick (2012)
Nostoc muscorum Agradh	Acetate + valerate + nitrogen-starved	P(3HB-*co*-3HV)	60	Bhati and Mallick (2012)

P: detected, but not quantified.

in biotreatment systems for wastewater (Laliberte *et al*., 1997).

Wastes such as swine manure, poultry litter, pressed mud, and sludge originating from the food and brewery industries and fishpond discharges contain high amounts of volatile fatty acids, which can be used as precursors of PHAs. The integrated efforts to clean up wastes and wastewaters using cyanobacteria will increase the ecological and economical benefits of PHA production by reducing the nutrient cost and relieving the burden on freshwater use as well. A few case studies from our laboratory utilizing wastes for PHA production are discussed below.

14.5.1 Aqua-waste utilization

Amongst agro-industries, aquaculture contributes significantly to the generation of wastewaters. Intensive aquaculture requires extensive use of feed inputs

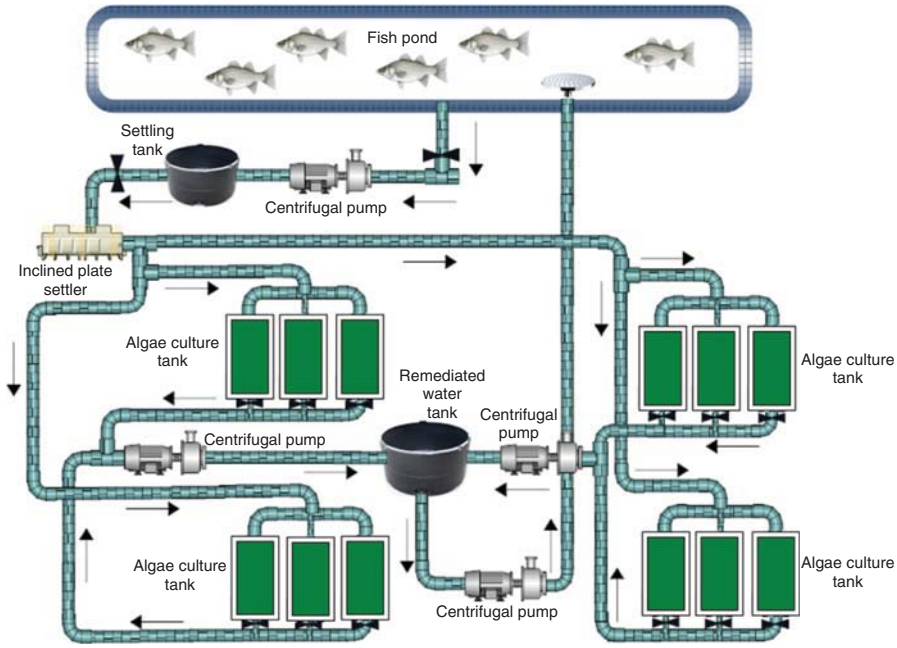

Figure 14.1 Diagrammatic representation of recirculatory aquaculture system. See plate section for color version.

that increase organic matter and nutrients, such as NH_4^+, NO_3^- and PO_4^{3-}, and suspended solids in ponds. They decrease the oxygen status and increase the turbidity of receiving waters (Lin and Yi, 2003). Due to environmental concerns and decreasing water availability, recirculation systems have emerged viable options for the aquaculture industry. We have developed a recirculatory aquawaste treatment system using *Aulosira fertilissima* to simultaneously produce PHB and remove the polluting nutrients from fishpond discharge (Figure 14.1).

The effect of sedimentation, inoculum size, mixing, depth, and seasonal variations on bioremediation and PHB production by *A. fertilissima* CCC 444 were studied in a recirculatory aquaculture system using fibre-reinforced plastic tanks (length $125 \times 60 \times 45$ cm). Under optimized conditions (Samantaray and Mallick, 2012), PHB yield varied between 60 and 69 g/l throughout the year. The bioremediation efficiency of *A. fertilissima* was maximum in the summer, followed by the rain season and winter. The level of dissolved oxygen increased in the range 3.2–6.9 mg/l during a culture period of 15 days. The concentration of nutrients such as ammonia, nitrite, and phosphate decreased to under the detection limit, demonstrating the bioremediation efficiency of the system and a high yield of cyanobacterial biomass for PHB extraction (Figure 14.2). The water quality parameters after 15 days incubation of *A. fertilissima* cultures were within the acceptable limits for aquaculture ponds (Hajek and Boyd, 1994; Srebotnjak *et al.*, 2012). Thus the data suggest a projected yield of ~17 t dry biomass, representing 14 t of PHB, annually per hectare, with simultaneous treatment of 32,640 m^3 of fish pond discharge (Samantaray, Nayak, and Mallick, 2011).

14.5.2 Poultry waste utilization

Poultry farming is another area that generates large amount of wastes. Effluents from poultry farms contain high concentrations of organic matter and inorganic nutrients, particularly nitrogen and phosphorus, both in soluble and particulate forms (Edwards and Daniel, 1992; Kelleher *et al.*, 2002). This carbon-rich waste could be a desirable feedstock for PHA production,

Figure 14.2 View of culture tanks with *Aulosira fertilissima*. See plate section for color version.

because mixotrophic growth conditions support high accumulations of PHAs (Table 14.4). In a photobioreactor study (Figure 14.3), different concentrations of poultry litter supplementation (5, 10, 15, and 20 g/l) stimulated the growth as well as PHB accumulation in *Nostoc muscorum* Agardh (Bhati, 2011). PHB accumulation was up to 23% (dcw) with 10 g/l poultry litter-supplementation, while the control value was 14% in BG-11 medium. On a substrate basis, PHB yield of 1.37 g/g poultry litter was recorded, which is significantly high compared to the yield reported for *Azotobacter vinelandii* UWD (0.29), *Pseudomonas cepacia* (0.15), and *Ralstonia eutropha* H16 (0.76 g PHB/g substrate) (Page and Cornish, 1993; Young, Kastner and May, 1994; Kahar *et al*., 2004). *Nostoc muscorum* filaments incubated in the medium supplemented with poultry litter produced about a 60% reduction in nutrient load after only a week of inoculation. NO_2^- was completely removed after 15 days, while NO_3^-, NH_4^+ and PO_4^{3-} concentrations were greatly reduced after 20 days.

14.5.3 Carbon dioxide utilization

Carbon dioxide accounts for 68% of total emissions of anthropogenic greenhouse gases. Producing PHA-based biodegradable plastics from carbon dioxide will certainly contribute to carbon neutrality. Bhati (2011) investigated the capability of *Nostoc muscorum* Agardh for carbon dioxide capture vis-à-vis PHA production. Carbon dioxide concentrations up to 10% (v/v) enhanced the growth of cyanobacterium significantly (1.1 g/l against 0.5 g/l control). The PHB yield obtained under 10% carbon dioxide-purging was around three times higher than that of the control. Both poultry litter supplementation and carbon dioxide-purging showed utilization of 3.27 g/g substrate (poultry litter), indicating that *N. muscorum* was more inclined to photoautotrophy during mixotrophic growth than heterotrophy for PHA production.

14.6 Surface study of PHA films

The upper surface of the PHA films has been analyzed by scanning electron microscope, revealing pores and protrusions that reduce considerably with incorporation of 3HV monomers. The PHB membrane shows a more rugged and porous surface. With 3HV content of 25% in the co-polymer, the size of protrusions and pores on the surfaces reduces, becoming nearly pore-free (Figure 14.4).

Figure 14.3 View of photobioreactor with *Nostoc muscorum* Agardh. See plate section for color version.

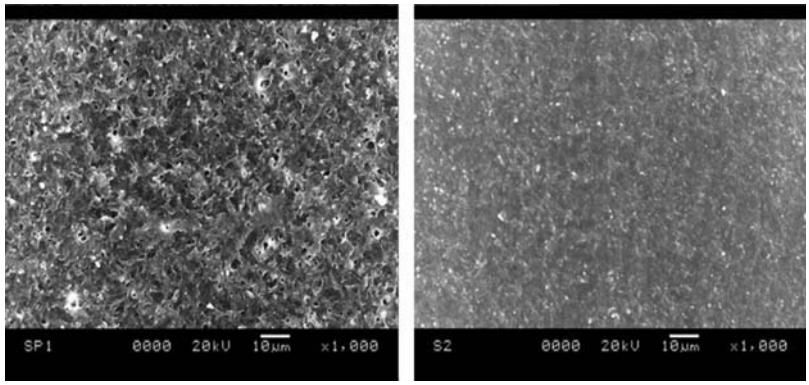

Figure 14.4 Scanning electron micrographs of the upper membrane surface of PHB and P(3HB-*co*-25% 3HV) films from *Aulosira fertilissima* (magnification 1000 ×).

14.7 Concluding remarks

Various studies indicate that cyanobacteria could be an alternative biological source for PHA production. Because of the phototrophic mode of nutrition and the nitrogen-fixing ability of certain species, cyanobacteria-based PHAs are likely to be cost effective and environment friendly. Wastewaters can be used for cyanobacteria-based PHA production. In addition, organic solid wastes (as media supplements) can also be used by certain species for enhancing PHA accumulation. Apart from providing a valuable product, ingeniously designed photobioreactors may help in bioremediation of contaminated environments and in carbon dioxide sequestration. To date, *Aulosira fertilissima* is the only cyanobacterium reported to accumulate PHB on levels at a par with bacterial species. Akiyama *et al.* (2011) have also demonstrated an appreciable amount of PHA accumulation by the recombinant *Synechococcus* sp. PCC 7002 (ATCC

27264). Therefore, further research efforts are needed to isolate/engineer efficient PHA-producing cyanobacterial strains, and to optimize their mass cultivation as well as the PHA-production and recovery processes to make "cyanoplastics" a commercial reality.

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PART IV
Harmful aspects

Chapter 15

Costs of harmful blooms of freshwater cyanobacteria

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15.1 Introduction

The occurrence of harmful cyanobacterial blooms (cyanoHABs) and their associated toxins tends to be intermittent (Oliver *et al*., 2012). Their distribution within a water body can be patchy, such that determining the occurrence and extent of blooms remains largely unpredictable (Oliver *et al*., 2012). As a consequence, assessing their economic impact is complicated, imprecise, and generally not well-suited to conventional valuation methods. The assessment of the economic consequences of cyanoHABs also raises important questions about how ecosystems are valued. One perspective is to approximate the economic value of provision of ecosystem services. A study by Costanza *et al*. (1997) of the economic value of ecosystem services from 16 biomes indicated that rivers and lakes yielded US $8,498/ha/year (at that time). Methods for assessing costs from eutrophication include "willingness to pay" (Dodds *et al*., 2009), externalities (Hearth, 1995), and a pressure–state–response framework (Pretty *et al*., 2003) based on two independently assessed costs relating to a reduction in the value of the asset (water) from:

- eutrophication-related effects
- the response in dealing with the adverse effects of eutrophication.

Because of the spatially and temporally sporadic nature of cyanoHABs, their economic assessments are inherently complex. Even when cyanoHABs have subsided there can be residual effects at an ecosystem scale arising from loss of biodiversity, reductions in the stability of the system, or as a result of persistence of cyanotoxins and associated breakdown products. Quantification of legacy effects is difficult to evaluate scientifically, let alone assess economically. Thus there is a large amount of imprecision in assigning a dollar value to the impact of cyanoHABs, particularly

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in attributing the costs resulting from the decline in ecosystem services.

There has been substantial investment in studies and monitoring of harmful marine algal blooms, prompted in part by the importance of biotoxins in commercial marine shellfish production (Hoagland and Scatasta, 2006). In contrast, studies on the economics in fresh water are limited and not as well-funded (Codd *et al.*, 2005), despite multiple adverse economic impacts, especially contamination of surface-water supplies.

The main ecological effects of cyanoHABs include deoxygenation of bottom waters arising from shading by dense cyanobacterial scums or from the oxygen demand created when mass aggregations of cells lyse, lose buoyancy, and sediment out of the surface water layer (Oliver *et al.*, 2012). These events can also smother the benthos, causing a decline in the diversity of benthic invertebrate fauna (Wang, Robertson, and Garrison, 2007). The more acute effects of cyanoHABs are associated with toxin production, which at least for some genera may be reinforced by aggregations of cells or colonies in surface blooms (Wood *et al.*, 2010). A detailed account of cyanotoxins is provided in Chapter 16.

Some toxin-producing genera form dense surface blooms (e.g., *Microcystis* and *Anabaena*), whilst others form sub-surface blooms (e.g., *Planktothrix* and *Cylindrospermopsis*). Benthic mat-forming species (e.g., *Phormidium*) are also becoming increasingly problematic in both standing and running waters (Heath, Wood, and Ryan, 2011; Wood *et al.*, 2012). The economic costs of cyanoHABs are mainly associated with these genera and relate to both the precautionary measures taken to avoid more severe impacts (e.g., closure or restriction of recreational access to water bodies) and the direct effects of toxins (e.g., stock deaths).

In this chapter, we categorize the different types of economic impacts that arise from cyanoHABs, provide information from studies across the globe that have attempted to quantify the monetary costs of blooms, and seek a blueprint for the future in terms of how economic costs may change with continued environmental pressures, as well as what might be done to mitigate these effects.

15.2 Categories of economic costs

Dodds and co-workers (2009) provided an economic assessment of the costs of eutrophication for freshwaters in the USA. They identified nutrients, particularly phosphorus, as the causal factor of eutrophication. Figure 15.1 shows a subset of factors considered by Dodds *et al.* (2009) in their eutrophication assessment,

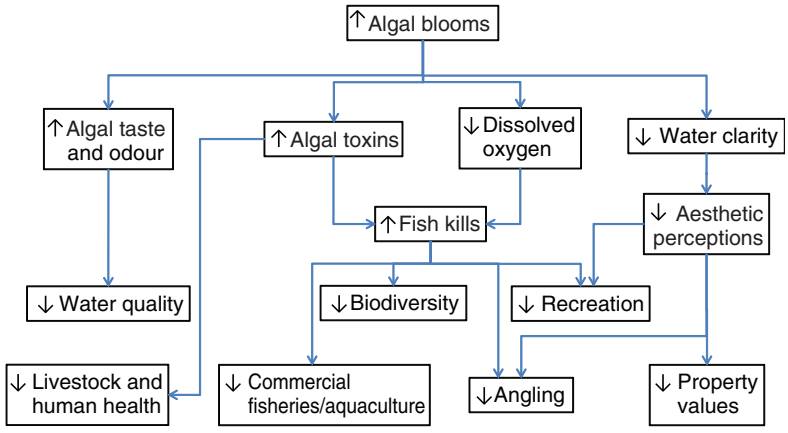

Figure 15.1 Categorization of economic costs of cyanobacterial harmful algal blooms for freshwater ecosystems (adapted with permission from Dodds, *et al.* 2009. Copyright © 2009, American Chemical Society).

which are relevant to our appraisal of the economic costs of cyanoHABs. The four categories of immediate effects arising from cyanoHABs are:

- occurrence of tastes and odors
- production of toxins
- depletion of dissolved oxygen
- and loss of water clarity.

The direct effects of cyanotoxins on human health are difficult to assess because of the wide range of responses induced by the different categories of cyanotoxins: neurotoxins, hepatotoxins, cytotoxins, and endotoxins. No monetary value has been assigned to human death or health impairment from cyanotoxin exposure. Furthermore, deaths of livestock and wildlife, which are intermittent and poorly documented, are mostly ignored in economic assessments (Pretty *et al.*, 2003; Dodds *et al.*, 2009).

15.2.1 Production of foul taste/odor compounds and toxins

Cyanobacteria produce a variety of chemicals such as geosmin (trans-1,10-dimethyl-trans-9-decalol) and 2-methylisoborneol that impart foul tastes and odors to affected waters. These chemicals impart earthy and musty odors to waters and are commonly perceptible to humans at concentrations of just a few nanograms per litre. The economic consequences of cyanoHABs and their by-products – cyanotoxins, and taste and odor compounds – in surface water supplies relate to five main areas:

1. increased costs from additional monitoring and surveillance in response to observations of cyanoHABs, with biovolume or cell concentration thresholds for cyanobacteria exceeded, or cyanotoxin concentrations reaching dangerous levels
2. increased levels of water treatment required to remove cyanotoxins to ensure the safety of public water supplies
3. specific treatments adopted for surface waters to reduce the immediate threat arising from cyanoHABs
4. identifying and remediating the causal factors responsible for bloom development, with a focus primarily on reducing nutrient inputs to surface waters
5. managing relevant scientific personnel and communicating information to the public to reduce health risks arising from occurrence of a cyanoHAB event, including information about cyanotoxins.

15.2.2 Surveillance and monitoring costs

Monitoring the costs of cyanoHABs is highly variable depending on the type of testing carried out and the extent of additional sampling required. Chorus and Bartram (1999) provide a comprehensive outline of monitoring requirements for toxic cyanobacteria in surface waters and place emphasis on the need for skilled personnel. Cell counts and taxonomic identification to species or genus level are generally required to provide reliable information on health risks. Public water utilities may be required to perform analytical toxin testing for accurate assessment of human health threats.

Steffensen (2008) opined that cyanoHABs could be relatively easily monitored in small isolated water reservoirs, with costs of a few thousand (Australian) dollars per annum. However, there may be many complexities to the monitoring of cyanoHABs in large, surface water reservoirs, including the high variability of populations (see Oliver *et al.*, 2012). These difficulties may be exacerbated by remoteness, multiple water offtake points, and exceptionally large areal coverage of blooms. For example, in China severe and persistent cyanoHABs have been reported for lakes Taihu (2,537 km^2), Dianchi (298 km^2) and Chaohu (775 km^2), which present major challenges for surveillance due to the high spatial variability of cyanoHABs and multiple offtake points for water supply (Le *et al.*, 2010). The most important aspect pertaining to the costs of cyanoHABs in these lakes relates to the closure of individual or multiple water treatment plant intakes. For instance, in events of severe cyanoHABs, all water treatment intakes were closed in Chaohu (in 1987) and Taihu (in 2007). The estimated costs of these closures exceeded RMB 100 million in each of the lakes

(Codd *et al.*, 2005; Le *et al.*, 2010). Similarly, the cost of a cyanoHAB in Lake Taihu in 1990 was estimated to be more than RMB 130 million (Yiping and Min, 2006). In the case of the 2007 cyanoHAB at Taihu, alternative water supplies, including bottled drinking water, were required for approximately 5 million people.

Atech (2000) have provided one of the few quantitative assessments of cyanoHAB surveillance costs, although these values are now somewhat outdated. They estimated that in Australia, AUD $8.7 million is spent annually on monitoring and contingency planning for cyanoHABs. It is rare to find itemized cyanoHAB monitoring costs that are independent from routine water-quality monitoring costs. For a single cyanoHAB (*Anabaena planktonica* bloom) event in the Waikato River (North Island, New Zealand), the monitoring cost was NZ $50,000 over three months during 2002–3 (Kouzminov, Ruck, and Wood 2007). During the most severe phase of the bloom, water supplies to around 1 million people were affected and alternative water supplies were contemplated for up to 150,000 people.

Dodds *et al.* (2009) made a comprehensive analysis of the costs of eutrophication damage to freshwaters in United States, but did not include the monitoring costs therein. Pretty *et al.* (2003) made no reference to cyanobacteria in their calculations of the direct costs of regulatory monitoring of water bodies in the United Kingdom, but included an estimate of £210,000 per annum for the additional costs of monitoring sensitive eutrophic water bodies. Lake Boehringen (0.735 km^2), a small meso-oligotrophic lake in southern Germany, had to be closed for camping, bathing, and all water recreational activities due to a heavy bloom of *Planktothrix rubescens* and concomitant high microcystin concentrations in 2011 and 2012. The monitoring cost was estimated at €10,000 per annum, while remediation efforts (installation of a solar-powered pumping device for artificial destratification), required an initial investment of €80,000 plus additional annual costs of €3000. The loss of income for the community due to the closure of the campsite was not detailed but may be around €10,000 (D.R. Dietrich, pers. obs.). These studies highlight the need to better assess the additional costs of cyanoHAB monitoring incurred by water management authorities, and to have a clear separation of these costs from those associated with routine monitoring of surface waters.

15.2.3 Cost of additional water treatment

The costs of additional treatment of water supplies to combat the effects of cyanoHABs include short-term contingency measures relating to provision of alternative supplies or providing additional temporary treatment and long-term measures such as arranging new water sources or upgrading or building new treatment plants (Kouzminov, Ruck, and Wood 2007). The occurrence of cyanoHABs is often a precursor to major investments in water treatment. For example following the 2002–3 cyanoHAB event in the Waikato River, a major water treatment plant upgrade was initiated, with a cost of NZ $5 million, in addition to costs for short-term contingency measures, including chemical treatment, of NZ $100,000 and up to $75,000 in capital works during the period of the bloom. According to a report by the Environmental Working Group, in the USA water bodies have required treatment designed specifically to remove taste/odor compounds and toxins associated with cyanoHABs (Naidenko, Cox, and Bruzelius, 2012). The report presents the costs of treatment plants for certain lakes as:

- US $8.5 million for Charleston, Illinois (21,000 people)
- US $15 million for Mattoon, Illinois (19,000 people)
- US $24.9 million for Mattoon, Illinois (100,000 people)
- US $31.8 million for Fairmont, Minnesota (11,000 people).

Dodds *et al.* (2009) alluded to the difficulty of separating additional water treatment costs arising from eutrophication from those incurred routinely during the treatment process. They used a "willingness to pay" assessment methodology based on estimates of the additional amount of money spent on bottled water to avoid taste and odor problems in municipal water supplies (i.e., from tap water). CyanoHABS represent a subset of the costs incurred from eutrophication, but are likely to be a major causal factor in the additional eutrophication treatment costs, which Dodds *et al.* (2009) estimated as US $813 million for the USA. Pretty *et al.* (2003) used an alternative approach in which 10% of water treatment operational

costs and 5% of capital costs were considered to be attributable to the additional costs incurred from treating cyanotoxins and taste/odor compounds. The assessment by Pretty *et al.* (2003) yields an additional treatment cost of £19 million per annum for the United Kingdom. A similar, but slightly higher value of AUD $65 million per annum has been assigned to Australia for the treatment of cyanoHABS for urban and rural extractive users (Atech, 2000). However, differences in methodologies and assessments between studies preclude a direct comparison.

15.2.4 Costs for treating and managing surface water supplies

A range of physical and chemical methods exist to deal with cyanoHABS in standing waters used for water supply. Direct applications of algicides such as copper sulfate have yielded mixed results. It resulted in hospitalization of more than 148 patients, mostly children, with symptoms of hepato-enteritis, following the treatment of a bloom of *Cylindrospermopsis raciborskii* in the major water supply reservoir in Palm Island (Queensland, Australia). The treatment resulted in mass lysis of cells, releasing cyanotoxins into the water supply (Hawkins *et al.*, 1985). Copper sulfate was used widely in water supply reservoirs in South Australia throughout the 1990s. Steffensen (2008) estimated that, including costs of copper sludge disposal, more than AUD $1 million per annum was spent on this treatment, both within the reservoir and in water treatment intakes. Thus there has been a move away from copper sulfate to the use of other compounds such as permanganate. Permanganate is a weak oxidant that is less likely to lyse cyanobacterial cells, but the cost of its use is nearly double that of copper sulfate (EPA, 2010). Another compound commonly used for the removal cyanotoxins is activated carbon. During a cyanoHAB in Ohio, the additional cost of intensive treatment with activated carbon reached $300,000 (EPA, 2010). There are various other methods for the effective removal of major cyanotoxins, such as slow-bank filtration, membrane filtration, or ultraviolet pre-treatment combined with advanced oxidation (Chorus and Bartram 1999), but they are expensive (Naidenko, Cox, and Bruzelius, 2012).

Aluminum sulfate ($KAl(SO_4)_2 \cdot 12H_2O$; alum) is considered to be a suitable compound for treatment of water affected by cyanoHABs, as it is not generally associated with release of cyanotoxins. However, Han *et al.* (2012) showed that alum treatment causes cell damage resulting in cyanotoxin release. The costs for alum treatment of surface water bodies are mostly embedded within the operational costs of water treatment with chemicals. For recreational lakes in the Rotorua region of North Island, New Zealand, chemical treatments have been used for aesthetic and recreational purposes to meet regional planning goals for water quality. Dosing with alum has been used mainly as a one-off application with the objective of controlling phosphorus so as to reduce cyanoHABs (Cooke *et al.*, 2005), but also on a more continuous basis by dosing lake inflows, which has reduced the risks associated with water acidification (Pilgrim and Brezonik 2005). Costs of alum treatment vary depending on many factors: the area and depth of the water body, the desired aluminum to phosphorus molar ratio and the characteristics of the water (e.g., hardness). Alternatives to alum have been used, such as dosing with modified zeolite (Aqual-P) or combinations of a phosphorus-binding clay (Phoslock®) and flocculation with alum or Phoslock® together with sediment dredging (van Oosterhout and Lürling, 2013). Abell *et al.* (2011) provided a figure of NZ $225,000 spent over three years on chemical treatment with Aqual-P, while the cost of treating Lake Rotorua (80 km^2) with alum was approximately $1 million per annum during 2011–2012 (Bay of Plenty Regional Council data).

Many management authorities have moved away from the use of chemicals to control cyanoHABs and cyanotoxins to physical methods such as artificial destratification, mechanical mixing, hypolimnetic oxygenation, hypolimnetic siphoning, and inflow diversions. Each has a slightly different mode of operation, either controlling cyanoHABs directly or indirectly, and they are applicable to both natural lakes and artificial storage facilities.

Artificial destratification is commonly accomplished by aeration and disturbance of the thermal stratification with rising bubble plumes, using aerators and mechanical mixers. The mixing results in replenishment of dissolved oxygen in bottom waters (Schladow and Fisher, 1995) and reduces anoxia-driven releases of nutrients from bottom sediments.

Artificial destratification can also alter phytoplankton assemblages, as the continuous mixing favors heavily silicified diatoms, which would otherwise settle out of the water column under quiescent stratified conditions, as well as being disadvantageous to cyanobacteria forming large colonies, such as *Microcystis* (Reynolds, 1984; Jungo *et al.*, 2001). The outcomes of destratification for water bodies dominated by smaller filamentous cyanobacteria (e.g., *Planktothrix* or *Cylindrospermopsis*) are less clear-cut and there is often little change in biomass despite achieving reduced chlorophyll concentrations (Antenucci *et al.*, 2005). A solar-powered artificial destratification device has been used to reduce *Planktothrix rubescens* blooms in Lake Boehringen (southern Germany), but it has an annual cost of €15,000 (D.R. Dietrich, pers. obs).

Surface mechanical mixers are designed to have a more direct effect on cyanobacteria. They prevent buoyant cells from accumulating and forming blooms at the water surface as well as potentially deepening the surface mixed layer. It must be emphasized here that artificial destratification works primarily in meso-oligotrophic surface waters when the process is continuously maintained. Mechanical mixers have been used in combination with artificial mixing by bubble plumes to provide complementary actions focused on reductions in nutrients and cyanobacteria (Brookes *et al.*, 2008). The cost of destratification (using mechanical mixers) varies with the desired extent of mixing and the size of water body. In a small reservoir in Perth (Western Australia) the monthly cost was AUD $30,000 (Kolman, 2001). Steffensen (2008) pointed out that large systems might have a capital cost of several hundred thousand (Australian) dollars, with running costs of approximately 10% of capital costs.

Hypolimnetic oxygenation is an alternative to destratification in which the objective is to achieve dissolution of dissolved oxygen injected directly into the hypolimnion, while preserving the stratification (Ashley 1993). Its action on cyanoHABs is less direct, mostly functioning through reducing nutrients released into the water column from bottom sediments. Beutel (2002) provided costs for different oxygenation systems of around US $1 million, and operating costs, mainly relating to supplies of liquid oxygen, of approximately US $1,000 per day or about 30% of capital costs.

Hypolimnetic siphoning is potentially a far cheaper alternative method of physical control. It aims to maintain a self-sustaining outflow of water from the hypolimnion of a lake. The success of this method depends on the extent to which the hydraulic residence time is altered, the oxygen demand in the hypolimnion, and changes in stratification and mixing due to deepening of the surface mixed layer. A combination of oxygenation and hypolimnetic siphoning installed in Lake Varese, Italy (14.5 km^2; mean depth, 10.7 m) strongly reduced the occurrence of CyanoHABs at a capital cost of US $4.3 million and operational costs of $150,000 per annum (Premazzi *et al.*, 2005).

In some cases it is possible to divert inflows away from lakes in order to reduce nutrient loads and the occurrence of cyanoHABs (Moss, Stansfield, and Irvine, 1991; Paul *et al.*, 2012). For Lake Rotoiti (34 km^2) which had a long history of cyanoHABs (Vincent, Gibbs, and Dryden, 1984; von Westernhagen, Hamilton, and Pilditch, 2010), a wall was constructed (at a cost of NZ $10 million) to divert a large, nutrient-rich inflow directly to the lake outflow (D.P. Hamilton, pers. obs.). The success of these types of measures is strongly dictated by the load of nutrients diverted relative to both the internal nutrient load and other external loads (Moss *et al.*, 1991).

In impounded river systems it is possible to influence both the biomass and the species composition using regulation of water flow between weirs (Mitrovic *et al.*, 2003). Critical transitions occur as flow velocity is increased, influencing stratification in the impounded water, from cyanobacteria (mostly *Anabaena circinalis*) to diatoms (*Aulcaoseira)* (Maier, Lee, and Wilkins, 2012). Steffensen (2008) provides examples of releases of water from dams of up to 70×10^6 m^3 but suggests that this method has been compromised in Australia due to the costs and value of water stored.

15.2.5 Management of catchment nutrient loads

The most sustainable way for long-term management of cyanoHABs is to reduce nutrient inputs to lakes and rivers by improving nutrient retentiveness in the catchment (Jeppesen *et al.*, 2007; Søndergaard *et al.*, 2007). Substantial costs may be involved in the different options designed to reduce nutrient exports, including

acquisition of land, consultation with stakeholders, and changes in land use or land-management practices (Carpenter *et al.*, 1998). In the United Kingdom, costs for schemes to decrease nutrient loads from agricultural sources to prevent eutrophication have been estimated at US $4.75 million per annum, which is small compared to an estimated cost of US $70.4 million per annum to upgrade wastewater treatment plants (Pretty *et al.*, 2003). Steffensen (2008) noted that a detailed assessment of costs of catchment management strategies is complicated by the extent to which these strategies are targeted towards preventing microbial contamination, aesthetic appearance, and eutrophication. Dodds *et al.* (2009) calculated the loss of property value due to eutrophication in the United States to be anywhere from US $14.1 to 141.1 billion per annum corresponding to assumed values of private ownership of land around the lakes of 5–50%, respectively.

As a consequence of reductions in nutrient load, lakes of various sizes may undergo slow re-oligotrophication (e.g. Lake Constance, Germany). Lakes with sufficient mixing and low hydraulic residence time returned to a near oligotrophic state, but other lakes, for example the pre-alpine lakes Zurich (Switzerland), Mondsee (Austria), and Ammersee (Germany) have much more prolonged stratification and thus are prone to gradual warming in conjunction with climate change, as well as blooms of filamentous cyanobacteria, such as *Planktothrix aghardii* and *P. rubescens*. The blooms may result, as in the case of Ammersee, in a significant reduction in fishery yields of coregonids (white fish) and thus in the endangerment of livelihoods associated with fishing enterprises.

15.2.6 Impairment of recreational activities and commercial fisheries

CyanoHABs can have severe direct impacts on freshwater fisheries as a result of fish kills (Barica, 1978), as well as indirect effects relating to accumulation of cyanotoxins (microcystins) in fish (Wood *et al.*, 2006) or shellfish tissue (Falconer, Choice, and Hosja, 1992). Pretty *et al.* (2003) estimated a loss of £29–118,000 per annum for commercial fisheries in the United Kingdom based on damages incurred from cyanoHABs. In the case of recreational activities, including fisheries, Dodds *et al.* (2009) estimated that eutrophication could lead to losses of up to US $1.16 billion per annum in the United States. Many tourism activities are closely linked to freshwater quality and clarity, and cyanoHABs therefore potentially compromise the viability of these enterprises.

15.3 Conclusions and future perspectives

The economic impacts of harmful freshwater cyanobacterial blooms may be related to direct water consumption to contact and non-contact recreational activities, including commercial and non-commercial fisheries. Ongoing environmental changes, including global warming, changes in land-use patterns to meet the food and shelter requirements of an expanding human population, and the increased translocations of organisms that accompany increases in mobility and global trade, are likely to increase the frequency and intensity of cyanoHABs, for the following reasons.

1. High air temperatures will lead to warming of water, in which cyanobacteria may thrive due to their ability to increase reproduction rates with increases in temperature faster than other phytoplankton taxa (Carey *et al.*, 2012; Winder, 2012). Paerl and Huisman (2008) have related global warming to the proliferation of cyanoHABs across the world. Of particular concern is the links between climate warming and increased lake stratification and the predominance of sub-surface species that occasionally form surface blooms, particularly the genus *Planktothrix* (Posch *et al.*, 2012).
2. Modern intensive agriculture increases the rate of export of nutrients from the landscape to water bodies, contributing to their eutrophication. Agricultural land use has been positively correlated with cyanobacteria populations in lakes (Paul *et al.*, 2012). Brookes and Carey (2011) suggest that catchment management designed to reduce nutrient loads could partially mitigate the effects of climate warming on cyanobacteria.
3. Invasive CyanoHAB species such as the toxin-producing *Cylindrospermopsis raciborskii* are likely to expand their distributions (Padisák, 1997). Their ability to thrive in a wide range of temperature and water-quality conditions, together with resistant

akinetes, facilitate their further spread as global connectivity increases (O'Neil *et al*., 2012).

Likely future increases in the incidence of cyanoHABs and the presence of cyanotoxins will require greater investments in catchment management, water and wastewater treatment, and surveillance. In the near future surveillance is likely to become increasingly sophisticated, although not necessarily cheaper, with the proliferation of telemetered systems that use remote sensors (Brient *et al*., 2008), PCR (Humbert, Quiblier, and Gugger, 2010), or fluorescence spectra (Alexander, Gikuma-Njuru, and Imberger, 2012) to provide information on cyanobacteria populations in real time, as well as level of cyanotoxins (Marion, Lee, and Wilkins, 2012). Modeling could be used to hindcast as well as forecast the occurrence of cyanoHABs (Robson and Hamilton, 2004; Harris *et al*., 2011).

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Chapter 16
Cyanotoxins

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16.1 Introduction

The cyanobacteria comprise a diverse group of oxygenic photosynthetic bacteria with equally diverse morphological features and physiological traits (Singh and Montgomery, 2011). Many cyanobacteria exhibit diazotrophy, often through the formation of specialized cell types, such as nitrogen-fixing heterocysts. This diazotrophy enhances the capacity of the cyanobacteria to produce a plethora of nitrogenous compounds (Herrero, Muro-Pastor, and Flores, 2001). Many of these compounds, including cyclic peptides and alkaloids, exhibit a range of biological activities, some of which are highly toxic to humans and other eukaryotic organisms (Neilan *et al.*, 1999).

This article discusses the economic implications of toxin production by aquatic cyanobacteria, predominantly within the context of freshwater reservoirs. We also present an overview of the various cyanobacterial bloom compositions and toxin profiles typically encountered in these ecosystems. When evaluating the true economic impact of cyanobacterial blooms, both indirect costs, such as those experienced by tourism, agriculture, and water suppliers, and direct costs, such as monitoring and treatment overheads, are considered.

16.2 Toxic cyanobacterial blooms

The rapid proliferation of cyanobacterial cells into toxic blooms poses a significant hazard for aquatic ecosystems worldwide. The increasing incidence and severity of bloom events has been associated with the ever-increasing anthropogenic eutrophication of urban and rural water bodies (Bowling and Baker, 1996; Sprőber *et al.*, 2003; Paerl, 1996). Whilst cyanobacterial blooms regularly occur within coastal and oceanic regions, their formation in inland freshwater reservoirs constitutes the greatest challenge to the water quality industry. This issue is compounded by the fact that released cyanobacterial toxins can cause water to be unsuitable for human consumption and recreational use for periods extending beyond the bloom cycle.

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In coastal, marine, and estuarine water bodies the implications of toxic cyanobacterial blooms are less severe than those faced in freshwater systems. Despite this, there are still a number of critical concerns facing coastal systems including the bioaccumulation of toxins in seafood and exposure as a consequence of recreational use (Miller *et al.*, 2010).

On a macroscopic level, many cyanobacterial blooms appear to comprise a single organism. However, when examined at a microscopic or molecular level these blooms represent complex microbial communities often containing more than one cyanobacterial species (Park *et al.*, 1993). This is complicated further by the presence of both toxic and non-toxic strains, of one or more species, the proportionate levels of which contribute to the final levels of toxins with the bloom (Al-Tebrineh *et al.*, 2012).

Over the last ten years, there has been an increase in reports of toxic cyanobacterial blooms from regions with no previous history of such events. At the same time the number of species identified as capable of producing toxins has increased. It is unclear as to whether this trend in the literature represents increased eutrophication, widening geographic distribution as a result of increased global traffic and climate change, or if it simply reflects increased monitoring regimes and the sensitivity of new detection protocols (Wagner and Adrian, 2009; Roelke *et al.*, 2012; Davis *et al.*, 2010; Burford and Davis, 2011, Paerl, Hall, and Calandrino, 2011). Whatever the case may be, the prevalence of toxic cyanobacterial blooms in freshwater supplies is undeniably placing great pressure on the global availability of our most important resource.

16.3 Cyanotoxins

Cyanobacteria produce numerous secondary metabolites that are not integral to their survival, but appear to offer some ecological or physiological advantage (Berdy, 2005; Neilan *et al.*, 2013). The cyanotoxins are a group of cyanobacterial secondary metabolites that exhibit activities detrimental to higher eukaryotic organisms including humans (Chorus *et al.*, 2000; Steffensen, 2008). The cyanotoxins span several chemostructural classes, but they are typically categorized based on their biological activity in humans (Sivonen, 1996; Haider *et al.*, 2003; Dittmann and Wiegand, 2006; Pearson *et al.*, 2010). In the following section we provide a brief overview of the major cyanotoxin toxocological subgroups, including the hepatotoxins, the neurotoxins, the cytotoxins, and the dermatoxins. The economic costs and potential risks associated with these molecules from both a historical and future projection viewpoint are also discussed.

16.3.1 Hepatotoxins

The hepatotoxins are a group of cyclic peptides that exhibit potent selective activity against hepatocytes (liver cells), through the inhibition of protein phosphatases (Honkanen *et al.*, 1990). The hepatotoxins are represented by two structurally and biosynthetically related classes; the microcystins and the nodularins (Figure 16.1). The microcystins are cyclic heptapeptides that feature a 3-amino-9-methoxy-2, 6, 8-trimethyl-10-phenyl-4, 6-decadienoic

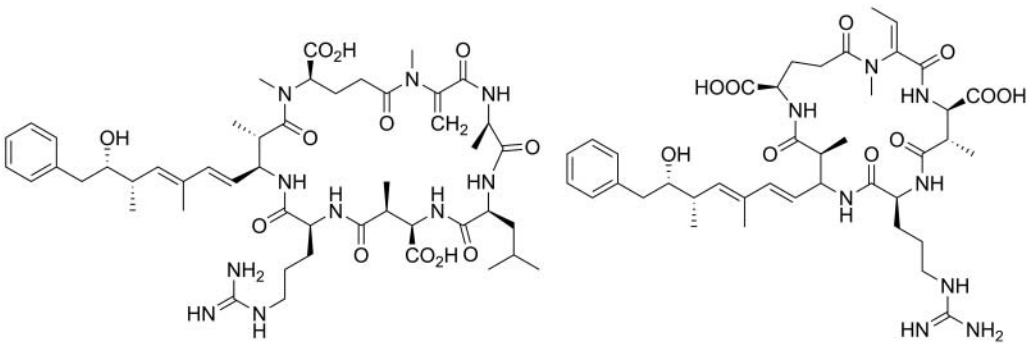

Figure 16.1 Chemical structures of cyanobacterial hepatotoxins, microcystin-LR (left) and nodularin (right).

acid (Adda) side group and two variable amino acid positions. These variable amino acids give rise to over 80 different structural isoforms, with each exhibiting a different toxicity profile (Tillett *et al.*, 2000; Pearson *et al.*, 2008). The nodularins are cyclic pentapeptides, structurally related to the microcystins in that they share the Adda moiety (Rinehart *et al.*, 1988). The only known nodularin isoform is due to a L-homoarginine (Har) residue being incorporated in the place of an L-Arg (Beattie, Kaya, and Codd, 2000; Saito *et al.*, 2001). Determination of the biosynthesis of both these hepatotoxins unveiled striking similarities, indicating that the nodularin biosynthetic pathway was derived from a deletion of the microcystin biosynthetic pathway (Moffitt and Neilan, 2004). Molecular probes targeting these pathways have since led to the identification of microcystin and nodularin biosynthesis in numerous genera including *Microcystis*, *Anabaena*, *Planktothrix*, *Nodularia*, *Nostoc*, and *Oscillatoria* (Pearson and Neilan, 2008).

16.3.2 Neurotoxins

Three families of alkaloid neurotoxins are produced by cyanobacterial species: anatoxin-a, anatoxin-a(s), and the paralytic shellfish toxins. A number of cyanobacterial genera can produce neurotoxins, including *Anabaena (Dolichospermum)*, *Oscillatoria*, *Cylindrospermopsis*, *Cylindrospermum*, *Lyngbya*, and *Aphanizomenon*.

The paralytic shellfish poisons are a range of hydroxylated and sulfated analogues derived from the carbamate alkaloid, saxitoxin (Humpage *et al.*, 1994). Saxitoxins (Figure 16.2) bind to and inhibit voltage-gated Na^+ channels in nerve cells leading to muscle paralysis, respiratory arrest, and suffocation (Rando and Strichartz, 1986; Strichartz *et al.*, 1986). Paralytic shellfish poisons are known to accumulate in marine shellfish, such as mussels, clams, and oysters (Humpage *et al.*, 1994). Anatoxin-a (Figure 16.2) and its structural homologue homoanatoxin-a act as potent agonists of the muscular and neuronal nicotinic acetylcholine receptor. Activation of this receptor leads to the increased flow of cations and the depolarization of the cell membrane. Because these toxins are not degraded, their prolonged presence sustains the desensitized state of the receptor and provokes muscle paralysis (rigid paralysis) and respiratory failure (Carmichael, 1994; Dittmann and Wiegand, 2006). Anatoxin-a(s) is physiologically and chemically distinct to anatoxin-a, and is an irreversible inhibitor of anticholinesterase, preventing the catabolism of acetylcholine (Carmichael, 1994). Anatoxin-a(s) provokes salivation (thus the suffix (s)), lacrimation, urinary incontinence, ataxia, and convulsions. Death is usually the result of respiratory collapse.

16.3.3 Cytotoxins

Cylindrospermopsin (Figure 16.3), a well known cytotoxin, is an alkaloid produced by several cyanobacterial species. Key producer organisms include *Cylindrospermopsis raciborskii* (Hawkins *et al.*, 1985) and *Aphanizomenon ovalisporum* (Banker *et al.*, 1997; Shaw *et al.*, 1999). Cylindrospermopsin incorporates a central guanidino moiety and a hydroxymethyluracil attached to a tricyclic backbone (Ohtani, Moore, and Runnegar, 1992). A single tautomic analogue of cylindrospermopsin – deoxycylindrospermopsin – has been isolated from *C. raciborskii* (Norris *et al.*, 1999), *Raphidiopsis curvata* (Li *et al.*, 2001), and *Lyngbya wollei*. In contrast to the original compound, deoxycylindrospermopsin was not lethal in an equivalent mouse assay (Griffiths and Saker, 2003). Cylindrospermopsin has been reported to exhibit cytotoxicity by targeting glutathione and protein

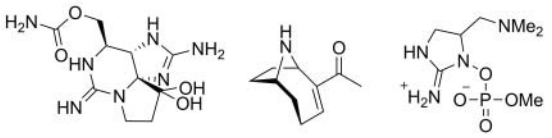

Figure 16.2 Chemical structures of cyanobacterial neurotoxins, saxitoxin (left), anatoxin-a (middle), and anatoxin-a(s) (right).

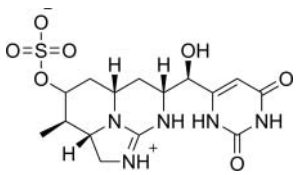

Figure 16.3 Chemical structure of the cyanobacterial cytotoxin, cylindrospermopsin.

synthesis pathways and by inhibiting cytochrome p450 (Froscio *et al.*, 2003, 2008b).

16.3.4 Skin irritants (dermatoxins)

Many reports of irritation to the respiratory tract and contact dermatitis in connection to cyanobacterial blooms have been made from both inland and coastal environments. In the 1970s incidences of contact dermatitis were reported by swimmers of Hawaii and Okinawa who had come into contact with the marine cyanobacterium, *Lyngbya majuscula* (Moikeha, Chu, and Berger, 1971; Hashimoto *et al.*, 1976). Extensive chemical evaluation led to the description of three tumorgenic compounds: aplysiatoxin, debromoaplysiatoxin (Mynderse *et al.*, 1977), and lyngbyatoxin (Figure 16.4; Cardellina, Marner, and Moore, 1979; Sims and Zandee van Rilland, 1981), which exhibit their action through the activation of protein kinase C (Fujiki *et al.*, 1990). Despite being described as skin irritants, there have been reports of the ingestion of the toxin or toxic cyanobacteria leading to gastrointestinal complications and death (Yasumoto, 1998).

16.4 Economic perspective

The economic costs associated with cyanobacterial toxins are those costs associated with monitoring, closures, and restrictions to water resources and access for agri/aquaculture and tourism, as well as costs associated with removal of the cyanotoxins and treatment of those individuals that have been exposed (Chorus and Bartram, 1999; Backer, 2002). In this way, if monitoring or early action is not implemented, negative health and economic implications associated with the refusal to address the problem early increase exponentially. For instance monitoring is an aspect that is generally considered cost-effective and allows for forewarning of any toxin production. If successful, a monitoring program can drastically reduce the economic costs associated with cyanobacterial blooms and their toxins by allowing for early intervention and prevention of exposure of individuals.

16.4.1 The economy of water

16.4.1.1 Drinking water

From a global perspective, access to fresh drinking water is a major cause for concern. In underdeveloped countries instances of toxic cyanobacterial blooms have resulted in several human fatalities (Azevedo *et al.*, 2002). Despite aspirations, access to clean fresh drinking water is still dependent entirely on a region's state of economic development. This is due, for the most part, to the extensive costs associated with monitoring, detection, and treatment regimes, to ensure good water quality. Amongst developed countries, whilst specific practices may differ, technologies utilized at each stage are typically consistent with "best practice" according to the World Health Organization. Under the specific guidelines for drinking water, only the microcystins are provided with a guideline level (WHO, 2011). This has forced many developed countries to set their own guidelines for toxins such as cylindrospermopsin, saxitoxin, and anatoxin (Table 16.1).

Toxic cyanobacterial blooms in water supplies can have major economic ramifications. Foremost is the potential health risk, which could see countless individuals requiring medical assistance due to exposure to cyanobacterial toxins. An additional risk factor for

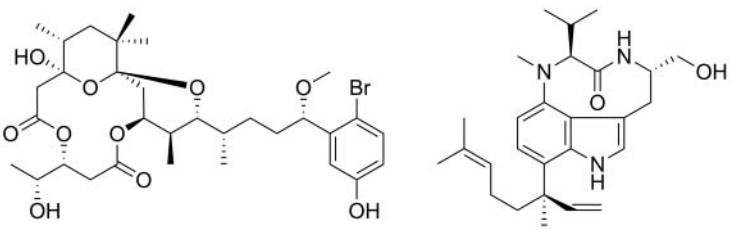

Figure 16.4 Chemical structures of the cyanobacterial irritant toxins, aplysiatoxin (left) and lyngbyatoxin (right).

Table 16.1 International guideline values for toxin concentrations within freshwater.

Toxin	Guideline value (μg/l)	Countries
Microcystin-LR	1.0	Czech Republic, China, France, Italy, Japan, Korea, New Zealand, Norway, Poland, Brazil, Spain
	1.3	Australia
	1.5	Canada
Nodularin	1.0	New Zealand
Saxitoxin	1.0	New Zealand
	3.0	Brazil
Anatoxin-a(s)	3.0	New Zealand
Anatoxin-a(s)	1.0	New Zealand
Cylindrospermopsin	3.0	New Zealand
	15.0	Brazil

Adapted from Newcombe, G., House, J., Ho, L., *et al.*, (2010) Management strategies for cyanobacteria (blue-green algae): A guide for water utilities, Water Quality Research Australia.

water managers is that of impending litigation due to a failure to act to secure clean drinking-water supplies. Large blooms in major water reservoirs, specifically in rural areas, require a large coordinated response, often involving sections of the military or police forces. Supplies of water from additional sources need to be rapidly organized, not just for drinking water, but also for ensuring supplies for maintaining hygienic practices.

16.4.1.2 Agriculture and aquaculture

Despite the emphasis placed on the consequences of toxic cyanobacterial blooms for freshwater drinking supplies, the implications for industries such as the agricultural and aquacultural sectors can be far more extensive. As the global population increases, more demands are being placed on a decreasing pool of natural resources including freshwater. Intensive practices, specifically in aquaculture, have been shown to lead to eutrophic conditions promoting cyanobacterial blooms.

While strict guidelines and monitoring regimes are in place for most drinking water supplies, water destined for industrial end uses is less stringently controlled. Early detection systems enable drinking water authorities to limit access to contaminated reservoirs, thereby intercepting the problem before it impacts on the health sector. Conversely, in industrial circles, inappropriate monitoring and unrestricted access to contaminated water can lead to the contamination of crops as well as the death of livestock (Hayman, 1992; Bowling, 1994; Thomas *et al.*, 1998; Saker, Thomas, and Norton, 1999). In circumstances where contaminated agricultural water is restricted, farmers are forced to pay premium rates to access alternative water supplies or face reduced crop/livestock yields.

The Murray-Darling basin in Australia represents a major drinking-water, industrial, and recreational resource, with many agricultural communities dependent on access to the water for irrigation. In recent years, drought conditions have led to industrial users extracting unsustainable quantities of water from the river, reducing flows. This in turn has resulted in an increase in the occurrence and severity of toxic cyanobacterial blooms, which in 2009 covered over 1100[hs]km of the Murray-Darling river (Al-Tebrineh *et al.*, 2012). Access to large portions of the river was limited due to the unknown diffusion of toxins through the river system. To the best of our knowledge, the combined economic costs of this bloom have not being determined.

16.4.1.3 Recreational uses

Many of the reservoirs utilized for drinking water and industry are also used for recreational activities, including fishing and water sports. Blooms in agricultural areas can therefore often impact on the recreational and tourism industries in affected regions as much as they affect primary industries. For example, in 1991 an extensive cyanobacterial bloom in the Darling River not only caused the death of livestock, but also cost the local tourism industry an estimated AUD $1.5 million (Steffensen, 2008). In general, zones dedicated to recreational activities are less stringently monitored than catchment areas used for drinking water. This is despite the fact that many of these activities involve direct contact with water, which increases the likelihood of being exposed to toxins (Chorus *et al.*, 2000; Codd, Morrison, and Metcalf, 2005).

Recreational exposure to cyanobacterial toxins can also occur in coastal and marine environments. This is typified by exposure of individuals to the skin irritants

lyngbyatoxin, aplysiatoxin, and debromoaplysiatoxin, which are produced by the marine cyanobacterium *Lyngbya majuscula* (Osborne, Webb, and Shaw, 2001). In the context of recreational fishing there is direct evidence for the bioaccumulation of toxins in seafood, which may have health consequences following ingestion (Humpage *et al.*, 1994; Araoz, Molgo, and Tandeau de Marsac, 2010). Moreover, an epidemiological study indicated that some allergenic and respiratory responses were attributable to contact, but not ingestion, of fish from cyanobacterial-infested waters.

Approaches to recreational use of contaminated water bodies are dependent on local government authorities educating end users. In many instances the visible scums and strong odors associated with toxic cyanobacterial blooms are an obvious indication of poor water quality and deter public use. In instances where bloom indicators are less obvious or where a bloom may affect the river system upstream, the most commonly used deterrent is signage. Whilst blooms are often viewed as short-term events, they can occasionally persist for months on end, or exhibit prolonged patterns of recurrence. In such cases, long-term damage to the local tourism industry may occur.

16.4.2 Direct costs

In reality the economic costs associated with toxic cyanobacterial blooms are extensive and impossible to fully quantify. As a result of a toxic cyanobacterial bloom, economic costs may impact on drinking-water providers, farmers, agriculture-dependent industries, tourism operators, and health providers, as highlighted in the previous sections. The next section of this chapter deals with the direct costs in the context of monitoring and detection, treatment, and finally health. It is important to note that in different countries and regions the available technologies may differ and, as such, associated costs will vary.

16.4.2.1 Monitoring and testing

The successful management of water bodies is dependent on the availability of accurate methods for testing and treating water. Water-quality protocols have evolved substantially over the past few decades, as increasing knowledge shapes new technologies. However, access to the latest test methods may be precluded by their high associated costs, forcing institutions to implement lower-cost alternatives. Despite this, governments recognize that downstream costs associated with poor water quality can be mitigated by performing routine monitoring and testing.

Routine water-quality evaluation is undertaken by water managers to assess a range of physicochemical parameters. When evaluating the risk, factors such as turbidity, stratification, nitrogen and phosphate levels, pH, and dissolved oxygen and chlorophyll a levels are considered. Evaluations are inexpensive and not considered an active part of the cyanobacterial monitoring process but can prove vital for predicting their formation (Meriluoto and Codd, 2005). In water bodies with a history of toxic cyanobacteria, where models prove accurate, access to water bodies can be restricted preventing harmful exposure. When predictions indicate no adverse risk, water accessibility is maintained to ensure no detrimental economic costs to stakeholders (NHMRC and NRMMC, 2011).

Microscopy has long been utilized as a tool for evaluation of cyanobacterial bloom samples, and this review would like to advocate its continued use in combination with modern molecular and analytical tools. In earlier years it was believed that the distribution of toxins was limited, but our current understanding indicates that there is a wide distribution of toxins amongst cyanobacteria. An additional shortfall of microscopic analysis is that some cyanobacteria exhibit morphological plasticity, leading to misidentification.

Animal toxicity assays enable both the qualitative and quantitative evaluation of toxin production. As a qualitative method these approaches can be used to determine whether an unknown toxic metabolite is associated with a cyanobacterial bloom. Whilst this approach circumvents some of the uncertainties associated with other methods there are some obvious shortcomings. These include, but are not limited to, low sensitivity, the effects of interactions, base level toxicity of cellular material and water, as well as ethical constraints. Moreover, they provide no indication as to which toxin isoform is being produced. As a quantitative tool, animal models can be applied when a known toxin is present to calculate toxicity equivalents per volume of water using data derived from LD50 determinations (Froscio *et al.*, 2008a). For laboratories

and organizations that contain in-house animal testing facilities associated costs are kept to a minimum. The majority of organizations, however, do not have these facilities and are forced to pay high rates for what is often considered specialized testing.

As a consequence of these high costs, *in vitro* methods for detection (which can be performed at a reduced cost with little expertise) have gained popularity (Meriluoto, Lawton, and Harada, 2000; Nicholson and Burch, 2001). Microcystin and nodularin detection and quantification is performed routinely using an *in vitro* assay that exploits the inhibition of protein phosphatases by these hepatotoxins (MacKintosh *et al.*, 1990; Lambert *et al.*, 1994; Serres, Fladmark, and Doskeland, 2000; Osborne, Webb, and Shaw, 2001). For other cyanobacterial toxins, molecular targets that can be transformed into commercially viable *in vitro* assays are not as effective. In these instances, enzyme-linked immunosorbent assays are utilized, with antibodies specific for the toxin molecule (Brooks and Codd, 1988). *In vitro* assays can be viewed as a method of preliminary screening, with multi-well plate formats allowing for high-throughput screening (HTS). The results of these HTS approaches can then be validated by techniques such as liquid chromatography/mass spectrometry or high-pressure liquid chromatography (HPLC; Sivonen, 2008). For *in vitro* assays non-specific binding may occur, leading to an overestimation of toxin quantification (Chu, Huang, and Wei, 1990; Honkanen *et al.*, 1994). This has led to a lack of confidence and limited use of these assays, especially when a false positive may result in the temporary closure of an entire industry.

Analytical methodologies are the gold standard for water-quality management. Thin-layer chromatography represents a simple and inexpensive approach for the isolation and detection of microcystins, but lacks the resolution of more advanced technologies (Harada *et al.*, 1988; Poon *et al.*, 1987). HPLC allows for ultra-high resolution, with detection possible at a picogram per injection level. When combined with a known standard, this method can be used for the simultaneous detection and quantification of multiple compounds and their analogues at high resolution. Even higher resolution can be achieved, for individual molecules, by using specialized separation columns and sensitive UV detectors (Lawton, Edwards, and Codd, 1994). When combined with tandem mass spectrometry, confidence is improved, with individual toxin analogues exhibiting mass-fingerprints following ionization (Bateman *et al.*, 1995; Lawton *et al.*, 1995; Yuan *et al.*, 1999a,b). Ultimately, there are significant costs associated with the analytical determination of toxins in the water supply. As such, these tests do not usually form part of routine water quality monitoring and testing regimes, but rather they are applied when the likelihood of a toxin being produced is considered to be high.

Molecular approaches may establish a new standard, in that they have the ability to provide information regarding what organisms are present, whether there is a genetic capacity to produce toxins, and how many toxic versus non-toxic cells are present within a given sample volume. Furthermore, recent studies have demonstrated a good correlation between the expression of toxin genes and the production of toxins, and therefore it is possible estimate the amount of toxin within a bloom using quantitative polymerase chain reaction (qPCR) (Al-Tebrineh *et al.*, 2010; Al-Tebrineh *et al.*, 2011). This technology comes with the additional advantage that it is inexpensive and has a short turnaround time. This means that this technology can provide near real-time information and is sufficiently cost-effective to feature as part of routine monitoring (Pearson and Neilan, 2008).

16.4.2.2 Treatment options

There are a number of preventative measures available that can reduce the likelihood and impact of cyanobacterial blooms. Many of these processes are not financially viable in that they involve education and planning rather than physical infrastructure or technology. Building on concepts outlined in the "Monitoring and detection" section of this chapter, a prior knowledge of the history of a given water body can be key. For instance, where water bodies have a history of toxic bloom events, a number of control measures can be put in place to prevent future blooms or reduce their impacts. These include, but are not limited to, restricting water use, reducing sources of pollution, and ensuring consistent flows are maintained to prevent stratification or nutrient enrichment (Page *et al.*, 2002; Paerl and Huisman, 2009; NHMRC and NRMMC, 2011; Posch *et al.*, 2012). Where appropriate, investments can be made to establish multiple water extraction points from areas within the reservoir

or water column where cell concentrations and toxin levels are reduced. Alternatively, the installation of devices that prevent stratification have proven very effective in limiting bloom formation (Jäger, Diehl, and Schmidt, 2008).

Toxins can be localized in both the intra- and extracellular space, but despite this, large quantities of toxins will only find their way into the water column following rupturing of the cell wall (Falconer, Beresford, and Runnegar, 1983). As a consequence of this, many treatment options act by preventing the dispersal of biomass and release of the toxins (Newcombe and Nicholson, 2004). Chemical flocculation is performed to promote settling and to enhance filtration-based processes. Filtration of cyanobacterial cells may have the negative consequence of contributing to cell lysis and toxin release, especially when high concentrations of biomass build up on membranes (Hall *et al.*, 2000). Activated carbon filtration (Cook and Newcombe, 2002) can then be applied to remove significant levels of specific toxins released prior to or as part of the primary filtration (Keijola *et al.*, 1988). The precise effectiveness of activated carbon filtration, when applied to a water treatment situation, remains disputed (Lahti and Hiisvirta, 1989; Lambert, Holmes, and Hrudey, 1996; Haider *et al.*, 2003). For this reason, other toxin removal processes should also be considered.

Oxidative ozonation of cyanobacterial toxins by the addition of chlorinated compounds allows for the degradation of some but not all toxins to non-toxic by-products. Whilst cylindrospermopsin, microcystin, and nodularin are readily oxidized at a nominal level of chlorine (Hitzfeld, Hoger, and Dietrich, 2000; Senogles *et al.*, 2000; Brooke *et al.*, 2006; Westrick *et al.*, 2010), saxitoxins and anatoxin-a are impervious to such treatments (Rositano, Nicholson, and Pieronne, 1998; Nicholson *et al.*, 2003). Whilst the process is cost effective, chlorination can only be applied to extracted water, with no options present for the addition of chlorinated substances to water reservoirs.

Bioremediation represents an additional option, specifically for the removal of microcystins, and can be applied to both extracted water and water reservoirs. Subsequent to bloom events, microorganisms have been isolated that are capable of biodegradation of microcystins. Initiated by the linearization of the cyclic peptide, degradation involves a number of enzymatic processes that are exhibited by a range of bacterial isolates. The addition of these enzymes into water reservoirs, in a controlled and rational way, provides an effective but costly method for removal of these toxic metabolites (Rapala *et al.*, 1994; Valeria *et al.*, 2006; Ho *et al.*, 2007; Kato *et al.*, 2007; Smith *et al.*, 2008).

16.5 Concluding remarks

The economic costs associated with toxic cyanobacterial blooms are complex and difficult to fully evaluate. What has been made clear in this review is that large economic impacts can be mitigated by first ensuring investment in low-cost monitoring regimes. Where blooms can be predicted with reasonable confidence and access to contaminated water is restricted, the economic costs can be maintained at low levels. Failure to implement such management strategies can lead to increased complications with associated economic and social implications.

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PART V

Tools, techniques, and patents

Chapter 17

Photobioreactors for cyanobacterial culturing

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17.1 Introduction

Cyanobacteria are amongst the most primitive forms of life on Earth. They are present in virtually all ecosystems (Carr and Whitton, 1982). Their cellular structure is typical of a prokaryote, even though they are larger in size than typical bacteria. They perform an oxygenic mode of photosynthesis, thus resembling plants. Many strains are diazotrophic, in other words they can use atmospheric molecular nitrogen rather than combined forms of nitrogen. They are able to use carbon dioxide as their sole carbon source via the (reductive) pentose phosphate pathway or the Calvin cycle (Stal and Moezelaar, 1997). Although mainly photoautotrophic, some cyanobacteria are non-phototrophic obligate heterotrophs as well as facultative heterotrophs capable of growing on organic substrates in the dark.

A wide range of applications of cyanobacteria has been described and discussed in the literature. Those that have attained commercial deployment encompass health foods (Anupama and Ravindra, 2000), food additives, pigments, diets for aquaculture, growth-regulating agents (Irisarri, Gonnet and Monza, 2001), sources of fluorescent tags (Glazer, 1994), as well as uses in wastewater treatment and the supply of secondary metabolites (Yamaguchi, 1996; Borowitzka, 1997). In addition, they may become a source of bioactive compounds as well as being used in carbon sequestration, biofuel production (Tsygankov *et al.*, 2002; Yoon *et al.*, 2002, 2006; Lee K.-S. *et al.*, 2004; Oh *et al.*, 2005), recovery of heavy metals from effluents, and even in outer-space technologies (Yamaguchi, 1996; Nagase *et al.*, 2001; Scragg *et al.*, 2002; Travieso *et al.*, 2002; Acién-Fernández *et al.*, 2005; Wang G. *et al.*, 2006).

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These prospects have made cyanobacteria one of the most promising organisms for biotechnology.

The development of cyanobacteria-based technologies is severely constrained by their relatively low volumetric productivities in industrial photobioreactors, both in terms of biomass and metabolite production rates. There have been several reports of development of photobioreactors and strategies specifically for cyanobacterial mass cultivation and the efficient production of valuable products (Suh and Lee, 2003; Kim J.D. and Lee, 2005; Kim Z.-H. *et al.*, 2006).

Closed systems (known as photobioreactors) suitable for cyanobacteria are very different from the fermenters designed for bacteria, yeasts, or fungi. Recent developments have tried to address issues such as efficient mixing, good light dilution and conduction, and membrane-mediated transport of gas. Industrial feasibility demands low construction and processing costs, but despite considerable effort, there is nothing like an optimal reactor system: one that is able to achieve maximum productivity at minimum operation cost for every microorganism selected and every final purpose intended.

From a commercial perspective, culturing of cyanobacteria and harvesting of products from them appear to have several advantages. These microorganisms are photoautotrophs, requiring only sunlight, water, and basic nutrients for growth. Moreover, unrestricted availability of exogenous carbon makes the large-scale culture of cyanobacteria in open ponds comparatively cheap (Apt and Behrens, 1999). Cyanobacterial species currently cultured in large ponds are extremophiles. They grow in highly selective environments that reduce the chances of contamination by other microalgae and bacteria. The final production cost hinges more on the biomass harvesting and product extraction than the production itself and therefore further advancements in downstream processing are required.

High biomass yields can also be achieved in fermenter-based systems (or photobioreactors) under vigorous agitation and with an effective light supply. Since cyanobacteria are structurally simple, processing of bioproducts is easier than where complex tissues are involved. Moreover, through use of genetic engineering, the recombinant proteins can be secreted directly into the culture medium in closed photobioreactors, or targeted for sequestering into the periplasmic space (Walker *et al.*, 2005).

The development of techniques to select and maintain particular strains of commercial interest that are difficult to grow in open-pond systems is at present a high priority for industry. Beyond the first few centimeters of depth, the supply of carbon dioxide, oxygen, and sunlight becomes limiting, and restricts bulk cell growth (Apt and Behrens, 1999). Specific surface area is a critical parameter affecting cell concentration and productivity. The need for turbulent flow to remove oxygen, and maintain access to sunlight and nutrient exchange adds to the complexity and cost of mass culture.

In this chapter, we describe the features of existing production systems for cyanobacteria, pinpointing the failings and describing opportunities for improvement.

17.2 Type of metabolism in cyanobacteria

For growth, cyanobacteria are critically dependent on supply of a carbon source, coupled with light to carry out photosynthesis (Moheimani, 2005). However, some cyanobacterial species can grow on certain substrates in darkness (facultative heterotrophs). Khoja and Whitton (1971) tested 24 species of cyanobacteria, representing 12 genera, using a basal inorganic liquid medium containing 0.01 M sucrose. Most of the species were able to grow in the dark, but at a lower rate than in the light. Similarly, Rippka *et al.* (1979) grew 178 strains of cyanobacteria, representing 22 genera, on a mineral-culture medium supplemented with fructose, glucose, or sucrose. Seventy-eight strains showed growth on at least one of these carbohydrates, and were classified as facultative photoheterotrophs. It is now established that some cyanobacteria can synthesize photosynthetic pigments and exhibit active photosystems during heterotrophic growth in the absence of light (Stanier and Cohen-Bazire, 1977; Kulik, 1995). Thus cyanobacteria show four distinct modes of nutrition: photoautotrophic, heterotrophic, mixotrophic, and photoheterotrophic.

Photoautotrophic cultivation occurs when cyanobacteria use light (e.g., sunlight) as an energy source and inorganic carbon (e.g., carbon dioxide) as a carbon source to store chemical energy via photosynthesis (Guedes and Malcata, 2011). This is the most

commonly used mode of cultivation for cyanobacteria (Gouveia *et al.*, 2009), and the only mode that meets the technical and economic criteria for large-scale production of biomass for purposes other than energy production (Guedes and Malcata, 2011). The approach appears particularly promising because cyanobacteria may in principle uptake carbon dioxide from the flue gases of factories and convert it to oil, thus providing a value to otherwise useless waste. Moreover, the chances of contamination are usually less than under heterotrophic growth (Mata, Martins, and Caetano, 2010).

Some cyanobacteria can use organic carbon (e.g., glucose) in the dark (Chojnacka and Noworyta, 2004) to support their growth. This type of heterotrophic cultivation avoids the problems associated with limited light availability, which precludes high cell densities under photosynthesis and permits a simpler scale-up process, as smaller ratios of reactor surface-to-volume are needed (Eriksen, 2008). Such systems are suitable for growth control, and also entail lower harvesting costs due to the higher cell densities attained (Chen G.-Q. and Chen, 2006). The set-up costs are low, although the system uses more energy than photoautotrophic cultivation because the whole cycle includes initial production of organic carbon sources via photosynthesis (Chisti, 2007). However, sugar-based heterotrophic systems are often sensitive to contamination (Carvalho, Meireles, and Malcata, 2006) especially, in open cultivation systems. This causes problems in large-scale production. In addition, from a commercial standpoint, the cost of organic carbon is a major concern. Scaling-up is less expensive than in photoautotrophic cultivation, making this approach technologically attractive, although not all chemicals can be produced heterotrophically (Degen *et al.*, 2001).

Mixotrophic cultivation occurs when a cyanobacterium undergoes photosynthesis, but resorts to both organic compounds and carbon dioxide as carbon sources for growth and metabolism. This means that it can live under either phototrophic or heterotrophic conditions, or both simultaneously. Cyanobacteria thus take up organic compounds and carbon dioxide, so the carbon dioxide released via respiration is trapped and reused under phototrophic cultivation (Mata, Martins and Caetano, 2010). Therefore, cell growth is no longer strictly dependent on photosynthesis, and light energy is no longer a limiting factor (Chen F. and Zhang, 1997). Growth rates of mixotrophic cyanobacteria are similar to those seen with photoautotrophic cultivation in closed photobioreactors, but the rates are higher or lower when compared with growth in open-ponds or under heterotrophic conditions, respectively (Brennan and Owende, 2010).

Photoheterotrophic cultivation takes place when cyanobacterial growth depends on light for energy production and organic carbon sources in conditions under which carbon dioxide fixation is impaired or non-existent (Zubkov, 2009). Although production of useful light-regulated metabolites can be enhanced via photoheterotrophic cultivation, this approach is seldom used to produce biomass (Guedes and Malcata, 2011).

17.3 Reactor design for biomass production

Cyanobacteria are efficient solar energy converters, and can produce a large variety of metabolites. However, this production potential remains largely unexplored (Ducat, Way, and Silver, 2011). Most commercial reactors for cyanobacterial culture are at present designed as open raceways (i.e., shallow open ponds in which culture is circulated by a paddle-wheel). Their technical and biological limitations have paved the way for the development of enclosed photobioreactors made of transparent tubes, sleeves, or containers, with natural or artificial light sources (Chaumont, 1993).

Cyanobacterial culture systems are generally classified according to their engineering (full or non-confining) and hydraulic characteristics. Because of their technical complexity, for a long time, photobioreactors were considered as the antithesis of open-pond technology, but the two approaches are now complementary for cyanobacterial mass culture. The gap between the theoretical biological potential of cyanobacteria and the actual biomass production could be narrowed in closed photobioreactors, which are more controlled.

At present, there are several reactor configurations available for mass cultivation of cyanobacteria. Their selection is based on factors such as the biology of the organism, the cost of land, the intensity of labor required, the cost of energy, availability of water, cost

of nutrients, suitability of climate (if the culture is implemented outdoors), and the specification of the final product(s). Large-scale culture systems can be compared using indicators such as their efficiency of light utilization, controllability of temperature, hydrodynamic stress allowable, ability to maintain unialgal and/or axenic cultures, and feasibility of scale up. These factors can help in the decision on whether to use closed or open ponds, indoors or outdoors, to cultivate cyanobacteria (Table 17.1).

17.3.1 Open systems

Cultivation of cyanobacteria in open ponds has been extensively studied (Boussiba *et al.*, 1988; Tredici and Materassi, 1992; Hase *et al.*, 2000). Open ponds are natural waters (lakes, lagoons, ponds) and artificial ponds or containers. The most commonly used systems include big shallow ponds, tanks, circular ponds and raceway ponds (Ugwu, Aoyagi, and Uchiyama, 2008).

In the early 1960s, circular ponds with large mixing arms were designed, but were difficult to scale-up to over 1000 m^2. Balloni *et al.* (1983) and Cardenas and Markovits (1985) proposed a board that encompasses the pond cross section, except for a slit of a few centimetres above the bottom, which is "dragged" through the culture pond. The culture is forced to run through the slit, thus promoting turbulent flow. Laws *et al.* (1986) described a mixing system consisting of a continuous flume containing arrays of foils, a design similar to the segments of airplane wings. They achieved average daily biomass production rates of over 40 g dwt/m^2 over periods of one month. Many other devices have been proposed to enhance mixing, such as air-lift (Persoone *et al.*, 1980), injectors, propellers, pump- and gravity-driven flow (Setlik, Veladimir, and Malek, 1970), and devices resorting to natural energy sources such as wind, sun, and even animals or humans (Becker and Venkataraman, 1982). Currently, the majority of open growing units are based on the raceway pond design originally proposed by Oswald (1969). It consists of long channels, arranged in a single or in multiple loops, coupled with stirring devices such as paddle-wheels (Chaumont, 1993).

Table 17.1 Major advantages and disadvantages of several culture types.

Culture type	Advantages	Disadvantages
Indoors	High degree of control (predictable)	Expensive
Outdoors	Cheap	Little control (less predictable)
Closed	Unlike contamination	Expensive
Open	Cheap	More likely contamination
Axenic	Predictable, less prone to crashes	Expensive, difficult
Non-axenic	Cheap, less difficult	More prone to crashes

Open ponds are easier to construct and operate than most closed photobioreactor systems. However, they are less efficient regarding biomass productivity than photobioreactors due to poor light utilization by the cells, evaporative losses, diffusion of carbon dioxide to the atmosphere, and the requirement for large areas of land (Chisti, 2007). Contamination by predators and fast-growing hetero- and photoautotrophs further restrict the commercial feasibility of open culture systems.

Those organisms that can withstand extreme conditions are better suited for open-pond cultivation. In open-pond systems, mixing is of great significance in terms of costs and productivity. Due to inefficient stirring mechanisms, in open cultivation systems mass transfer rates are poor, causing low biomass productivity. The recent advances in open culture system technology include the development of mixing systems specifically to prevent sedimentation and to enhance light utilization efficiency. Moreover, biomass productivity is not only dependent on the total amount of solar energy impinging on the culture surface, but also on the quantity of energy available at the cell level. The concepts of the "light regime" and "light per cell" were carefully considered by Richmond (1987). The chief advantages and limitations of open ponds are summarized in Table 17.2.

Temperature fluctuations owing to diurnal cycles and seasonal variations are difficult to control in open ponds (Chisti, 2007). The carbon dioxide requirement is usually supplied from the open atmosphere on its surface, but submerged aerators may be installed to enhance carbon dioxide absorption (Terry and Raymond, 1985).

In outdoor cultures, photosynthetic cells are subjected to diurnal illumination of varying intensity. The photon flux density varies by more than

Table 17.2 Major advantages and disadvantages of culture systems.

Culture systems	Advantages	Disadvantages
Open pounds	Relatively economical, easy to clean up after cultivation, good for mass cultivation of cyanobacteria	Little control of culture conditions, difficulty in growing cyanobacterial cultures for long periods, poor productivity, large occupancy of land mass, limited to few strains, cultures easily get contaminated
Vertical-column photobioreactor	High mass transfer, good mixing with low shear stress, low energy consumption, high potential for scalability, easy to sterilize, readily tempered, good for immobilization of cyanobacteria, reduced photoinhibition and photo-oxidation	Small illumination surface area, sophisticated materials required for construction, shear stress to cultures, decrease of illumination surface area upon scale-up
Flat-plate photobioreactor	Large illumination surface area, suitable for outdoor cultures, good for immobilization of cyanobacteria, good light path, good biomass productivity, relatively cheap, easy to clean up, readily tempered, low oxygen build-up	Scale-up requiring many compartments and support materials, difficulty in controlling culture temperature, some degree of wall growth, possibility of hydrodynamic stress to some strains
Tubular photobioreactor	Large illumination surface area, suitable for outdoor cultures, fairly good biomass productivity, relatively cheap	Gradients of pH, dissolved oxygen and CO_2 along tubes, possibility of fouling, some degree of wall growth, requirement for large land space

300 mol photon/m^2/s within a single hour for most of the day. It is consistently observed in outdoor cultures that the photosynthetic conversion efficiency is high in the early morning, but drops suddenly before mid-morning to about one quarter of the previous level. Low overall productivity in outdoor photosynthetic cultures has been attributed to their inability to rapidly respond to fast increases in photon flux density (Lee Y.-K. *et al.*, 1995).

The control of contaminants in open-pond systems is most important. Use of simple plastic covers or greenhouses over the open ponds has resulted in improved biomass productivity (Richmond *et al.*, 1993). On the other hand, Tamiya *et al.* (1953) found that these factors produced little reduction in contamination. More importantly, problems such as capital costs, maintenance, overheating, and difficulties in creating large units limit the applicability of covered systems. Four basic approaches have been proposed to maintain monocultures in open ponds (Benemann, Tillett, and Weissman, 1987; Richmond, 1987):

1. Use of selective conditions that favour the growth of cyanobacterium to be cultured (*Spirulina* is a good example). In future, genetic recombination may lead to an increased tolerance of cyanobacteria to extreme conditions.
2. Culture of cyanobacterial strains suitable for open ponds, which may not produce a high-value metabolite.
3. Maintenance of sterile or clean culture conditions as long as possible during scale-up, management of culture to avoid contaminant development and, in the worst situations, chemical treatment of contaminants to destroy fungi or zooplankton.
4. Development of closed photobioreactors (Richmond, 1992).

Despite several limitations, open pounds are still the most widely used systems at an industrial level. An example is *Spirulina* cultivation, where microbial contamination can be minimized via highly selective operating conditions, namely high pH. Unfortunately, high pH, temperatures, and salt concentrations are unsuitable for many commercially useful cyanobacteria (Guedes and Malcata, 2011).

Several technological improvements will be required for cyanobacteria to become competitive in existing markets (Dismukes *et al.*, 2008). One pressing concern is to design cost-effective bioreactors (Melis, 2002; Huber, Iborra, and Corma, 2006; Rupprecht *et al.*, 2006; Posten, 2009; Ducat, Way and Silver, 2011). Open systems have apparently reached their efficiency limits, with little room for

further technological improvements. To overcome the problems and limitations of open ponds, attention has now focused on developing suitable closed systems, such as flat-plate, tubular, vertical-column, and internally-illuminated photobioreactors.

17.3.2 Closed systems

Closed photobioreactors are mainly designed to overcome the problems associated with open-pond production systems (i.e., contamination, harvesting, and light sourcing); (Metting, 1996; Acién-Fernández *et al.*, 2001; Ugwu, Aoyagi and Uchiyama, 2008). They support high yields of cyanobacterial biomass (Narwade, Rajmalwar and Shrivastava, 2011). High performance photobioreactors should be of an universal design suitable for cultivation of various cyanobacteria. Efficient use of light by culture requires uniform illumination of the culture surface and fast mass transfer of carbon dioxide and oxygen.

Cyanobacterial cells are highly adhesive, resulting in rapid fouling of the light-transmitting surface, this necessitates frequent shutdown of the photobioreactor for mechanical cleaning and sterilization. An effective reactor design should prevent (or at least minimize) fouling, particularly of the light-transmitting surface. During high rates of mass transfer foaming occurs, and a photobioreactor should function normally under such conditions. Photobioreactors are of three basic types: tubular (Tredici and Materassi, 1992; Lee Y.-K. *et al.*, 1995; Hai *et al.*, 2000), flat plate (Kwon, Rögner, and Rexroth, 2012), and the fermenter-type (Carvalho and Malcata, 2001). The first two are specifically designed for efficient recovery of sunlight whereas the last may require artificial illumination.

A typical photobioreactor is essentially a four-phase system, consisting of the (solid) cyanobacterial cells, the liquid growth medium, the gaseous phase, and the superimposed light-radiation field (Posten, 2009). Therefore, a rational understanding of the complex interaction between biomass production and associated environmental parameters (e.g., fluid dynamics and light transfer) within the reactor is required. Various design features limit the productivity of a closed photobioreactor. The reactor has to be operated under favourable illumination, with optimized surface-to-volume ratio and light/dark cycle, coupled with adequate mass-transfer features (Tsygankov, 2001). The intensity and wavelength of the incident light are important, as they determine light dilution, light attenuation, and light mixing throughout the system (Uyar *et al.*, 2007; Tamburic *et al.*, 2011).

Enclosed photobioreactors provide reproducible cultivation conditions such as good heat transfer, give better biomass yield, higher product quality, and also an opportunity for flexible technical design (Borowitzka, 1999; Ugwu, Aoyagi and Uchiyama, 2008). They are more appropriate for sensitive strains due to the tight control over contamination that is possible. Moreover, the high biomass levels produced in close photobioreactors reduces the harvesting costs significantly (Chaumont, 1993). However, the other costs of closed photobioreactors is very high compared to their open-pond counterparts (Carvalho, Meireles and Malcata, 2006). A variety of closed photobioreactors have been proposed for cyanobacterial biomass production, and these have been comprehensively reviewed elsewhere (Molina-Grima, 1999; Tredici, 1999).

There is a consensus among biotechnologists that commercial-scale (photoautotrophic) production of high-value metabolites from cyanobacteria requires outdoor enclosed photobioreactors (Borowitzka, 1996; Tredici, 1999; Pulz, 2001). Scaling up of photobioreactors from laboratory to commercial scales is more of an engineering problem than a biological one. It requires changes in illumination, gas transfer, and temperature (all affected by turbulence within the reactor), as well as requiring good control. Much work has been done to describe the light field inside photobioreactors (Molina-Grima *et al.*, 2000; Olaizola, 2003). Photobioreactors such as the bubble-column, airlift, and stirred-tank types have good scalability, but their use outdoors is limited due to the reduced illumination surface areas they provide (Table 17.2); (Ugwu, Aoyagi and Uchiyama, 2008).

Despite higher volumetric productivity, closed systems are not a consensual industrial choice because of the high capital investment required and the high production costs. To minimize production costs, the major factors playing a role in the process need to be identified and quantified (Guedes and Malcata, 2011). The

choice of system depends on the objective. For instance, wastewater treatment would preclude closed systems owing to the high costs that arise from the large volumes to be processed and the low added value of the resulting products (Carvalho, Meireles, and Malcata, 2006).

17.4 Operation mode

17.4.1 Batch systems

A typical batch culture consists of a single inoculation of cells into a bioreactor containing the culture medium. Under optimal and sub-optimal conditions (temperature, pH, and aeration), cyanobacteria go through all growth phases (lag, exponential, stationary) over a period of several days. Harvesting takes place when the population reaches its maximum (or near-maximum) density. In practice, cyanobacteria are transferred to larger culture vessels prior to reaching the stationary phase, are allowed to reach maximum density, and are then harvested. Then, after cleaning and sterilization the fermenter is ready for another batch.

Because of their simplicity and flexibility, batch-culture systems are widely used. This allows species to be changed and defects to be remedied rapidly. Although often considered as the most reliable method, batch culture is not necessarily the most efficient one. Batch cultures are normally harvested prior to the onset of the stationary phase, and have to spend a substantial time growing at the maximum specific growth rate. The quality of the harvested cells is less predictable than in continuous systems, and varies with the timing of the harvest (e.g., the time of the day). Moreover, there is a strict need to prevent contamination during inoculation and the early growth period. Since the density of the desired microorganism is low and the concentration of nutrients is high, any contaminant with a faster growth rate will outgrow the intended cyanobacterium. Batch culturing is labour intensive (Table 17.3).

Studies have been carried out to optimize culture conditions and/or production of a given compound in batch cultures. Sinetova *et al.* (2012) studied the batch culture dynamics of *Cyanothece* sp. ATCC 51142 to find the dominant constraints during the individual growth phases, and to determine the possibility of inducing or delaying transitions between culture growth phases in terms of nitrogen fixation. This cyanobacterium holds a remarkable potential for the production of third-generation biofuels, but this varies with the time of day and the phase of culture growth. Other studies have tackled:

- the optimization of hydrogen photoproduction and carbon dioxide uptake by *Anabaena variabilis* (Markov, Bazin, and Hall, 1995; Markov *et al.*, 1997)
- the kinetics of nitrogen and organic matter removal from parboiled rice effluent by *Aphanothece microscopica* Nägeli (Queiroz *et al.*, 2007)
- determination of carbon dioxide fixation rates and lipid production by *Synechocystis aquatilis* TISTR8612 by determining its optimal growth conditions in batch mode using airlift photo-bioreactors (Kaiwan-arporn *et al.*, 2012).

Table 17.3 Advantages and disadvantages of modes of operation.

Culture type	Advantages	Disadvantages
Batch	Easiest to set up, most reliable, versatile, safe in terms of sterilization, complete conversion of substrate, environmental conditions easy to control	Least efficient, inconsistent quality, skilled labor required, large idle time in sterilization, growth of inoculum and cleaning after fermentation, and safety problems when filling, emptying, and cleaning
Semi-continuous	Easier, somewhat efficient	Laborious, less reliable
Continuous	Efficient, constant product quality, possible automation, highest rate of production, low labor cost, good utilization of reactor	Difficult, complex, high equipment costs, often disappointing, inflexible, difficult to control

17.4.2 Semi-continuous systems

Semi-continuous cultivation is a viable alternative system in which a portion of the culture medium is periodically removed, and the remaining culture used as starting point to carry on the culture. This ensures a high inoculum ratio at the time of addition of new medium. Semi-continuous cultures are characterized by dilution cycles with the same duration as cell cycles, and provide a simple tool to ascertain the characteristics of cultures maintained at different light–dark cycles, avoiding the disturbance that sometimes occurs under continuous dilution (Otero *et al.*, 1998). Semi-continuous cultivation also has operational advantages, such as keeping a constant amount of inoculum and allowing the cultured microorganism to maintain high specific growth rates (Table 17.3); (Fábregas *et al.*, 1996).

The semi-continuous technique extends the use of large tank cultures via partial periodic harvesting, followed by topping up to the original volume and supplementation with nutrients to achieve the starting level of enrichment. Both indoor and outdoor operations are possible. However, the duration is unpredictable, as is the impact of competitors, predators and/or contaminants, and metabolites eventually build up, rendering the culture unsuitable for further use. Since the culture is not completely harvested, this method yields more biomass than the batch method for a given tank size.

Although semi-continuous cultivation has often been used to grow cyanobacteria, this type of system is not well studied. There is lack of scientific data on biomass concentrations and renewal rates. Reichert, Reinehr, and Costa (2006) studied the semi-continuous cultivation of two *Spirulina platensis* strains in a closed photobioreactor to assess biomass concentration and renewal rate. They reported that lower blend concentrations and higher renewal rates favour fast growth rate and productivity. The maximum specific growth rate attained was four times that in batch cultivation, while the productivity was double.

17.4.3 Continuous system

In continuous cultivation, fresh medium flows into the fermentor continuously, and a part of the medium in the reactor is withdrawn from the fermentor at the flow rate similar to the inlet flow. Microorganisms are therefore placed in an essentially constant environment, with fixed feed rates to and from the system, and with a steady supply of limiting substrate and other nutrients. Consequently, over time, microorganisms can adjust their enzyme levels, pH, osmotic gradients, and macromolecular composition for "optimal growth". This situation is generally referred to as the steady state and may take up to 10–20 generations to attain.

The two major categories of continuous cultures are:

1. turbidostat culture, in which the biomass concentration is kept at a preset level by diluting the culture with fresh medium by means of an automatic system.
2. chemostat culture, in which fresh medium is introduced into the culture at a steady but predetermined rate, thereby adding limiting vital nutrient at a fixed rate; growth rate rather than cell concentration is kept constant.

In general, continuous cultivation has several advantages over batch cultures: constant production (photosynthesis) rates under defined cultivation conditions (Posten, 2009), high productivity compared to alternative modes, automation, and reduction in time for cleaning and sterilization (Donati and Paludetto, 1999). Efficient control over cell density and medium composition is important for the optimization of process parameters, as these depend strongly on culture density. Keeping all relevant parameters constant enables optimization of individual factors (Czitrom, 1999). Moreover, data from continuous cultures are much more straightforward to interpret, as they are in a dynamic equilibrium (or at most involved in small excursions from the steady state), and cause-and-effect relationships are more obvious (Kwon, Rögner, and Rexroth, 2012).

The major disadvantages of continuous systems are their relatively high cost and complexity, with continuous production intended to last long periods (say months) failing due to infection or through the spontaneous mutation of the microorganisms to non-producing ones. Moreover, the system is quite inflexible, and can seldom be used for other products without substantial retrofitting. The requirements for

constant illumination and temperature restrict continuous systems to indoor operation, which is feasible only for relatively small production scales (Table 17.3). Several attempts have been made to cultivate cyanobacteria under continuous culture systems (Weiss, Grome-Elhanan, and Halmann, 1985; Javanmardian and Palsson, 1992). Chetsumon *et al.* (1994) described the continuous culture of *Scytonema* sp. TISTR 8208 in a seaweed-type bioreactor to accomplish stable antibiotic production.

17.5 Processing parameters

At the industrial level, cultivation of cyanobacteria is mainly for food, pigment, and nutraceutical production (Olaizola, 2003). Besides sunlight and carbon dioxide, such production requires a nitrogen source and minor amounts of silicon, phosphate, and sulphate. Sea water might be used to cultivate cyanobacteria, since this contains ionic forms of nearly 70 elements including sodium, chloride, magnesium, potassium, sulphate, and calcium (Matsunaga *et al.*, 2005). However, extensive research is needed before developing such a system.

Several methods have been described for the optimization of the medium and the cultivation process (Li *et al.*, 2008; Wang B. and Lan, 2011), and the harvesting and biomass processing (Singh, Nigam, and Murphy, 2011). Important factors involved in cultivation/processing are temperature, gas transfer, pH, nutrients, light, agitation, building material, and the possibility of immobilization.

17.5.1 Temperature

Temperature is an important factor affecting cyanobacterial biomass composition, and strongly influences its oxygen-evolving activity. It also affects cyanobacterial membranes and influences nutrient availability and uptake (Murata, 1989; Inoue *et al.*, 2001; Vonshak, 2003b). Extreme temperatures reduce biomass production. The optimal temperature for phytoplankton growth lies in the range 20−30°C; temperatures below 16°C slow down growth, whereas those above 35°C are normally harmful (Hanagata *et al.*, 1992; Andersen and Andersen, 2006; Graham, Graham, and Wilcox, 2008). The optimum temperature to support cyanobacterial growth varies with genus and strains (Talbot *et al.*, 1991; Vonshak, 2003b). It is 35°C for *Anabaena variabilis* (Fontes *et al.*, 1987) and 30–38°C for *Spirulina* sp. (Rafiqul *et al.*, 2003; Vonshak and Tomaselli, 2003; Ogbonda, Aminigo, and Abu, 2007). Some *Spirulina* sp. strains are even thermophilic (Murata, 1989). Studies indicate that with increasing temperature protein content decreases (Talbot and de la Noue, 1993), carbohydrate content increases (de Oliveira *et al.*, 1999), and lipid content and biomass composition varies (de Oliveira *et al.*, 1999; Ogbonda, Aminigo, and Abu, 2007; Abu, Ogbonda, and Aminigo, 2007; Colla *et al.*, 2007). The highest crude protein content was achieved in *Spirulina* sp. at 30°C, at a pH value of 9.0.

There is an interrelation between temperature, light, and photoinhibition. At low temperatures, cyanobacteria are photoinhibited by high light intensities. Temperature is therefore an important limiting factor in outdoor cultivation during winter. However, photoinhibition considerably reduces upon temperature increases (Jensen *et al.*, 1993; Vonshak, 2003a,b), and some cyanobacteria (e.g., *Synechococcus*) can tolerate temperatures up to 60°C (Miyairi, 1995). The ratio of oxygen to carbon dioxide solubility increases with temperature, promoting oxygen fixation via oxygenase activity of the RuBisCO. Moreover, affinity of the ribulose-1,5-bisphosphate carboxylase oxygenase for carbon dioxide decreases upon temperature increases. To overcome these problems, cooling methods are often used to keep the culture within a favourable range, for example submersion of the entire reactor system in a water pool, spraying with water, shading, or providing a heat exchanger (Mehlitz, 2009).

17.5.2 Gas transfer

Gases introduced into bioreactors serve a number of purposes (Pulz, 2001):

- supply of carbon dioxide
- stirring stirring in order to expose all cells to light
- control of pH
- removal of excess dissolved oxygen.

Typically, cultures are re-circulated with the aid of a mechanical pump or airlift system, fine bubble

diffusers, or jet aerators and aspirators. The last of these allows exchange of carbon dioxide and oxygen between the liquid medium and the aeration gas, as well as providing a path for mixing (Eriksen, 2008). Amongst the alternatives, bubbling of carbon-dioxide-enriched air into the bottom of the bioreactor with bubble diffusers is the preferred approach.

Carbon dioxide enrichment and/or oxygen removal greatly improve the performance of cyanobacterial cultures. Due to photosynthesis (especially when its rate is high) oxygen accumulation takes place. The majority of the available techniques are inefficient in removing oxygen from cyanobacterial cultures. In tubular photobioreactors oxygen accumulates during the loop cycle and can be removed only by a degasser (Weissman, Goebel, and Benemann, 1988). High levels of dissolved oxygen are toxic to cyanobacteria because, under intense solar radiation, reactive oxygen species may form from dissolved oxygen causing cell-membrane damage and many other deleterious effects.

Carbon dioxide is the main carbon source for cyanobacteria in photoautotrophic culture, and may be limiting if carbon dioxide concentration is low in the injected gas or when mixing is not sufficient. A high concentration of dissolved carbon dioxide results in low pH, which inhibits growth. To maintain an optimal balance between dissolved carbon dioxide and oxygen, an overhead space for gas exchange is included in a photobioreactor. Mixing is effective in promoting turbulence and favours mass transfer between the gas and liquid. It also promotes chemical reactions between carbon dioxide, water, and ammonia, thus affecting the availability of carbon dioxide.

17.5.3 pH

Cyanobacteria mainly favour neutral-to-alkaline pH (7–9). *Anabaena* sp. displays optimal growth at pH 7.4–8.4 and its productivity decreases significantly at pHs above 9 (Fontes *et al.*, 1987; Vieira, Colla, and Duarte, 2004). However, there are species with optimum pH of around 4 (*Chlorococcum littorale*; Kodama, Ikemoto, and Miyachi, 1993) and also of 8–10 (*Spirulina platensis*), with poor growth at pH 7.0.

There exists a complex relationship between carbon dioxide concentration and pH in cyanobacterial bioreactor systems (Sanchez *et al.*, 2003), largely due to the chemical equilibrium among chemical species such as carbon dioxide, H_2CO_3, HCO_3^- and CO_3^{2-}, which is crucial to maintain the culture pH in the optimal range. Increased carbon dioxide concentrations may lead to higher biomass productivity to a certain extent, but concomitant decreases in pH may adversely affect the cyanobacterial physiology. Dissolution of carbon dioxide results from a balance between mass transfer of carbon dioxide from the gas to the liquid phase, and consumption of carbon dioxide by culture cells. In open ponds, microalgal growth causes a rise in pH to 10–11 (Oswald, 1988). This increase in pH may prove beneficial for inactivation of pathogens but can also inhibit the growth of the intended organism.

The speciation of NH_3 and NH_4^+ in bioreactors is strongly dependent on pH. Ammonia uncouples electron transport in cyanobacterial photosystems and competes with water molecules in oxidation reactions, leading to the release of oxygen (Hu, Zarmi, and Richmond, 1998). Dissolving SO_x rather than carbon dioxide from the flue gas can influence the pH of the culture medium (see Maeda *et al.*, 1995 and Westerhoff *et al.*, 2010 for detail). A pH control mechanism should be integrated with the aeration system by adding basic medium to the culture. This is a standard practice in conventional bioreactors.

17.5.4 Carbon and nitrogen source

Carbon is an essential nutrient for the cultivation of cyanobacteria. Nearly 50% of the microalgal biomass is made of carbon (Becker, 1994), and approximately 1.8 kg of carbon dioxide is needed to produce 1 kg of biomass (Amaro, Guedes, and Malcata, 2011). In addition to organic sources, carbon can be utilized from organic compounds such as sugars (Lodi *et al.*, 2005), fatty acids, and amino acids. However, the take up of organic compounds under heterotrophic or mixotrophic growth is species-dependent (Chen F., Zhang, and Guo, 1996; Chojnacka and Noworyta, 2004; Muhling, Belay, and Whitton, 2005). *S. platensis* grows well in high-strength organic artificial wastewater medium containing acetate (Ogbonna, Yoshizawa, and Tanaka, 2000). Moreover, mixotrophic cultures (also less susceptible to photoinhibition) show improved growth rates

compared to autotrophic and heterotrophic cultures. Mixotrophic cultures require integration of both photosynthetic and heterotrophic components during the diurnal cycle. There is a reduction in biomass loss during (dark) respiration, with a decrease in the amount of organic substances utilized during growth.

A cheap source of carbon dioxide to fuel cyanobacterial photosynthesis is needed (Wang B. *et al.*, 2008). Cyanobacteria can be used to withdraw carbon dioxide from three major sources: carbon dioxide from the atmosphere, carbon dioxide emission from power plants and industrial processes, and carbon dioxide from soluble carbonates (Wang B. *et al.*, 2008). Mitigation of atmospheric carbon dioxide is probably the most basic method to sequester carbon, and relies on its mass transfer from the air to organisms in their aquatic growth environments during photosynthesis (Wang B. *et al.*, 2008). However, atmospheric carbon dioxide (0.03% v/v) is not sufficient to support the high cyanobacterial growth rates and productivities needed for full-scale biofuel production (Stepan *et al.*, 2002). Because of the high carbon dioxide concentrations (15–20%), carbon dioxide from flue gases emanating from industrial or power plants running on fossil fuels gives better results (Bilanovic *et al.*, 2009; Chiu *et al.*, 2009). In regular production facilities, carbon dioxide is injected into the growth media from external sources (Brown, 1996; Kadam, 2002; Doucha, Straka, and Livansky, 2005; Vunjak- Novakovic *et al.*, 2005; Hsueh, Chu, and Yu, 2007).

Cyanobacteria may assimilate carbon dioxide from soluble carbonates such as Na_2CO_3 and $NaHCO_3$ (Colman and Rotatore, 1995; Emma *et al.*, 2000; Wang B. *et al.*, 2008). Moreover, due to the high salt content and resulting high pH of the medium, it will be easy to control invasive species, as a limited number of cyanobacteria (and microalgae) can grow under such extreme conditions (Wang B. *et al.*, 2008). In aqueous media, inorganic carbon exists in different chemical forms – carbon dioxide (aq), H_2CO_3, HCO_3^-, and CO_3^{2-} (Goldman, Dennett, and Riley, 1981) – which are reversibly transformed via reactions controlled by pH and temperature (Becker, 1994). Reactions that interconvert carbon dioxide, H_2CO_3, HCO_3^-, and CO_3^{2-} in soluble form are fast. The synthesis of the enzyme carbonic anhydrase is enhanced when cells are exposed to a low-carbon-dioxide environment. HCO_3^- is easily absorbed by cells, but is a poor source of carbon compared to carbon dioxide (Markl, 1977; Goldman, Dennett, and Riley, 1981).

After carbon, nitrogen is the second most limiting nutrient for biomass production. Cyanobacteria normally utilize nitrates, but some are capable of utilizing urea or ammonia as nitrogen sources (Becker, 1994; Hsieh and Wu, 2009). Cyanobacteria can be used to remove nitrogen (also phosphorus) from municipal, agricultural, and agro-industrial wastes (Mata, Martins, and Caetano, 2010; Singh and Gu, 2010; Amaro, Guedes, and Malcata, 2011; Giorgos and Dimitris, 2011). Filamentous cyanobacteria are best suited for this goal because they attain high biomass levels and can be harvested easily owing to their size and structure (Giorgos and Dimitris, 2011). Certain cyanobacteria can utilize NO_x (Welsh *et al.*, 2000; Moreno *et al.*, 2003).

Sulphur and phosphorus are other elements required for cyanobacterial growth (Celekli, Yavuzatmaca, and Bozkurt, 2009). Phosphorous must be supplied in excess because phosphate ions bind to metals ions, decreasing their bioavailability (Chisti, 2007). Iron and many other trace elements are also required for the growth of cyanobacteria.

17.5.5 Light

High light intensities for long periods cause photoinhibition, which may be reversible or irreversible (Rubio *et al.*, 2003; Torzillo *et al.*, 2003). Both the utilization efficiency and overall photosynthetic efficiency depend on the nature of the incident light and the saturation threshold (Torzillo *et al.*, 2003). Exposure to natural photosynthetically active radiation produces high biomass, but this is reduced by up to 40% by accompanying UV radiation (Gao, Yu, and Brown, 2007).

For outdoor cultivation, the diurnal photo cycle is important (Pulz and Scheinbenbogan, 1998). This varies with season, and can strongly affect the overall efficiency of solar energy capture. In the absence of light, cyanobacteria have to resort to respiration to support metabolism and to maintain cell viability. Up to 42% biomass produced during daytime may be lost the following night (Tredici *et al.*, 1991; Jacob *et al.*, 2009). Barbosa *et al.* (2003) have demonstrated that long light–dark cycles may have negative effects on

cyanobacterial growth and light utilization, but this effect is eliminated by short time exposures (e.g., 10 s). Biomass yield and growth rate also depend heavily on the light fraction.

Artificial lighting permits continuous production, but demands a significantly higher energy input. Fluorescent lamps are commonly used for large-scale cyanobacterial cultivation (Muller *et al.*, 1998), but prior to choosing an artificial light source it is necessary to determine the absorption spectra of the major accessory pigments. Light limitation due to the thickness of the top layer reduces biomass productivity, but inclined culture systems and improved mixing can minimize the loss (Setlik, Veladimir, and Malek, 1970; Pulz, 2001; Carlsson *et al.*, 2007; Chisti, 2007; Ugwu, Aoyagi, and Uchiyama, 2008). Reduction in the length of the light path and improved mixing are the most effective methods to improve light distribution.

17.5.6 Agitation

Mixing of cyanobacteria cultures is necessary. It ensures that all cells are uniformly exposed to light and nutrients, prevents sedimentation of cells, facilitates heat transfer and avoids thermal stratification, and improves gas exchange. Agitation can be accomplished by aeration, pumping, and mechanical stirring (using rotation wheels or static mixers), or a combination of these approaches. Mechanical agitation and bubble break-up cause hydrodynamic stress, inhibiting cyanobacterial growth and metabolic activity (Suh and Lee, 2003; Vunjak- Novakovic *et al.*, 2005). Agitation also minimizes the deleterious effects of high light intensity (Hu, Guterman, and Richmond, 1996; Carlozzi and Torzillo, 1996) and, if combined with a flashing light, increases biomass productivity up to 40% (Ugwu, Ogbonna, and Tanaka, 2002). Inadequate agitation permits clumping of cells into aggregates of varying sizes, and these may lead to the formation of a three-phase system (gas/liquid/solid) inside the photobioreactor, which hampers mass transfer (Panda *et al.*, 1989). On the other hand, strong agitation leads to shear-induced injury to cells (Thomas and Gibson, 1990; Gudin and Chaumont, 1991; Camacho *et al.*, 2000). In sparged cultures agitated at low intensities, bubble break-up at the liquid–gas interface and bubble formation at the sparger causes maximum cell damage (Barbosa, Hadiyanto, and Wijffels, 2004).

17.5.7 Building material

The building materials of photobioreactors are important from the standpoints of cost-effectiveness and light-capture and gas-transfer efficiencies. Common materials used for photobioreactors are glass, plexiglas (Tredici *et al.*, 1991), PVC, acrylic, and polyethylene. Flexible PVC tubing is frequently used, but these become brittle due to UV damage. Glass is inert and transparent and suitable for small-scale photobioreactors, but in large-scale units the additional requirement of multiple connection parts makes it costly.

Building material surfaces are prone to formation of biofilms, which are difficult to clean off and which reduce light transmission drastically. In general, the choice of building materials and the construction design require careful consideration as regards light transmission, lifespan, and re-usability.

17.5.8 Immobilized cells

Immobilization of cells on artificial surfaces (e.g., kappa-carrageenan, alginate, calcium alginate) is preferred for the removal of nutrients from urban wastewater (Chevalier and De la Noue 1985; Mallick, 2002, Alejandro *et al.*, 2010). There are a few advantages over free cells, such as an increase in cell retention time within the bioreactor and metabolic activity therein (Lukavski, 1986; Tam, Lau, and Wong, 1994; Jimenez *et al.*, 2004).

17.5.9 Strain selection

Selection of appropriate culture strains is key to any cyanobacterium-based process (Sheehan *et al.*, 1998; Rosenberg *et al.*, 2008; Bruton *et al.*, 2009). Desired characteristics of a strain are high photosynthetic efficiency resulting in high biomass production, high metabolite productivity, robustness to shear stress and environmental factors, favourable competition with contaminant bacteria or microalgae, basic nutrient requirements, easy harvesting, and a richness in value-added metabolites (Pulz and Scheinbenbogan, 1998; Ono and Cuello, 2006). As is obvious, no strain can meet *all* these requirements, and therefore site-specific strains are the key to commercial success (Sheehan *et al.*, 1998). Both isolation of local strains with

specific traits and genetic manipulation of potential strains are needed (Dunahay, Benemann, and Roessler, 1998). On the basis of their size (around 200 μm) filamentous cyanobacteria are better suited for harvesting than unicellular forms (0.5–30 μm; Molina *et al.*, 2003; Olaizola, 2003; Waterbury, 2006).

17.6 Harvesting techniques

Harvesting refers to the concentration of a diluted cyanobacterial suspension as a thick dry paste. It accounts up to 20–30% of the total processing costs, and includes electricity, reagents, and maintenance (Borodyanski and Konstantinov, 2002; Mata, Martins, and Caetano, 2010). Typical harvesting is a two-step process:

1. Bulk harvesting of large biomass volumes, with concentration ~90-fold to attain 2–7% thick paste. The exact percentage obtained depends on the initial biomass concentration and methodology employed, including gravity sedimentation, flotation, or flocculation.
2. Use of techniques such as centrifugation, filtration, and ultrasonic aggregation to concentrate the slurry; this is generally more energy-intensive than bulk harvesting.

Selection of an appropriate technology is crucial for economic feasibility (Schenk *et al.*, 2008). For instance, *Spirulina*'s long spiral shape (20–100 μm long) makes it suitable for the cost- and energy-efficient microscreen harvesting method (Benemann and Oswald 1996). The basic strategy of harvesting is to apply first the processes that could reduce large volumes, followed by more selective procedures.

17.6.1 Centrifugation

Centrifugation is the preferred method of harvesting at industrial scales. It is more appropriate for harvesting high value-added metabolites and to produce concentrates with extended shelf lives (Heasman *et al.*, 2000). The process is fast but energy-intensive. The degree of recovery depends on the settling characteristics of the cells, the slurry residence time in the centrifuge, and the settling depth (Molina *et al.*, 2003). High harvesting efficiencies (above 95%); (Heasman *et al.*, 2000), and high concentration (up to 150-fold for an original 15% total suspended solids) can be achieved (Mohn, 1980) The chief disadvantages are the high energy and maintenance costs (Bosma *et al.*, 2003). In large-scale culturing systems a major concern during centrifugation is the high throughput of a large fraction of water (Golueke and Oswald, 1965).

17.6.2 Filtration

Filtration is carried out on membranes of modified cellulose, with the help of a suction pump. The main advantage is the ability to collect cells at very low concentrations (Borchard and Omelia, 1961). However, this technique is feasible for small volumes only. The conventional filtration process is appropriate for harvesting of relatively large (i.e., above 70 μm) cells, but is scarcely useful at sizes around 30 μm (Mohn, 1980). Conventional filtration operates under pressure or suction; diatomaceous earth and/or cellulose can improve the efficiency (Molina *et al.*, 2003). To recover small cyanobacteria, membrane microfiltration and ultra-filtration are viable alternatives to conventional filtration (Petrusevski *et al.*, 1995), although this precludes harvesting fragile cells that require a low trans-membrane pressure and low cross-flow velocity (Borowitzka, 1997). Improvements such as reverse-flow vacuums, and direct vacuums with stirring blades above the filter (to prevent particles from settling) are being applied to make the process more efficient, as well as to enable larger volumes of slurry to be handled and over shorter time periods (Danguah *et al.*, 2008).

17.6.3 Settling

Settling (gravity-driven sedimentation) is a common harvesting method, and is used to separate large-scale suspensions (Nurdogan and Oswald, 1996). However, it is suitable only for large cell sizes (larger than 70 μm; Munoz and Guieysse, 2006). The settling velocity can be enhanced by increasing the cell dimensions, in other words by aggregating cells into larger clumps (Schenk *et al.*, 2008); chemical coagulants are used for this

purpose. Moreover, centrifugal forces can also be used to increase the settling velocity.

The stability of cyanobacteria in the growth medium is mainly associated with the cell size, surface charge, and density. The higher the stability, the lower will be the tendency to settle. Electrical interactions of opposite charges between cells, and of cells with the surrounding culture broth, contribute to destabilization of the suspension (Tenney *et al.*, 1969).

17.6.4 Flocculation

Flocculation is often the first step in the harvesting of cyanobacteria and is intended to aggregate cells to increase their effective particle size. Note that the cell surface of cyanobacteria usually carries a negative charge that prevents spontaneous aggregation of cells in suspension. Flocculants block these surface charges (i.e., decrease or neutralize the negative charge) by binding at high pH, allowing cells to adhere to each other and generate flocks. Flocculants may also physically bind to more than one cell via a process called bridging (cross-linking) that facilitates aggregation prior to filtration, flotation, or sedimentation (Molina *et al.*, 2003). Biomass is separated from large volumes of water more easily by flocculation and sedimentation than by filtration (Palmer, 1978; Divakaran and Pillai, 2002).

Flocculants that have met with success include chitosan (Divakaran and Pillai, 2002), aluminum salts, polyacrylamide polymers (Morales, dela Noiie, and Picard, 1985), alum (hydrated aluminum potassium sulphate), lime (Folkman and Wachs, 1973), alkalis (Dziubek and Kowal, 1989; Lee, 1998; Semerjian and Ayoub, 2003; Knuckey *et al.*, 2006), and such multivalent metal salts as ferric chloride, aluminum sulphate, and ferric sulphate. Aluminum salts are widely used for sewage dewatering and removal of cyanobacteria from drinking water (Nonomura, 1987) but are undesirable for animal feed unless the aluminum is removed afterwards. Knuckey *et al.* (2006) reported a process that entails manipulation of pH between 10 and 10.6 using sodium hydroxide followed by the addition of Magnafloc LT-25 (a non-ionic polymer). The flocculate was then recovered by siphoning off the surface water after a settling period and subsequent neutralization, with overall efficiencies close to 80%. Adjusting the culture pH (Sukenik *et al.*, 1985) and bio-flocculation (Golueke and Oswald, 1965) are alternatives to chemical additives.

Flocculation is typically followed by either flotation or sedimentation. The latter is easier in older cultures; otherwise forced flocculation may be needed to promote sedimentation. To induce flotation, air is bubbled through the cell suspension causing cell clusters to float to the surface. Subsequently the top layer is removed (Parker, 1975). Ami *et al.* (2012) reported an inexpensive non-toxic method of flocculation of cyanobacteria using calcium hydroxide or magnesium hydroxide: the former is preferable because of its lower cost and use of calcium as a nutrient in animal feed (besides precipitating more cells).

17.6.5 Flotation

Flotation involves trapping cells using dispersed microscopic air bubbles (Wang B. *et al.*, 2008). Some strains float naturally (Bruton *et al.*, 2009), depending on their lipid content. Froth flotation can be used to separate biomass from the original suspension by manipulating pH and bubbling air through a column, thus creating froth that accumulates above the liquid level, from where it can be removed by suction. Froth flotation and subsequent drying is currently considered as too expensive for commercial use, yet flotation is a flexible low-cost operation (Liu, Chen, and Ju, 1999).

Following the addition of cationic surfactants, dissolved air flotation effectively separates cyanobacteria from water (van Vuuren and van Duuren, 1965; Funk, Sweeney, and Proctor, 1968; Bare, 1975; Liu, Chen, and Ju, 1999). *Microcystis* was eliminated via an ozone-flotation method (Benoufella *et al.*, 1994). Pure-oxygen aeration was poor at flotation, unlike ozone, which required a range of 0.005–0.03 mg/mg biomass, making it a promising option for harvesting. Unfortunately, ozone is a strong oxidant that normally oxidizes unsaturated and aromatic rings into carbonyls and carboxylic acids, respectively (Beltran, 2004; Li Y.-Q. *et al.*, 2008; Von, 2003), which may affect the quality of the metabolites that will be extracted downstream from the cyanobacterial biomass.

17.6.6 Electrophoresis

Electrophoresis involves the field-induced motion of particles relative to a fluid. It can be used to separate biomass from its medium and it does not require the addition of chemicals. In this technique, an electric field drives the negatively charged cyanobacterial cells out of the suspension (Mollah *et al.*, 2004). If, in addition, electrolysis is carried out, the hydrogen released from the water adheres to the cell flocks and carries them to the surface. The advantages of using electrochemical methods include environmental compatibility, energy efficiency, safety, versatility, and selectivity (Mollah *et al.*, 2004). Alfafara *et al.* (2002) conducted an investigation of the removal of cyanobacteria electrolytically in batch and continuous reactors coupled with flotation. Meanwhile, Bosma *et al.* (2003) successfully used ultrasound to standardize aggregation efficiency with a 92% separation efficiency and a 20-fold concentration factor. The main advantage of ultrasonic harvesting is that it is suitable for continuous operation without inducing the shear stress that could damage some value-added metabolites, besides its intrinsic non-fouling nature (Bosma *et al.*, 2003). Electro-flocculation, on the other hand, has proven to separate around 95% of cells from fresh water (Poleman, de Pauw, and Jeurissen, 1997).

17.7 Concluding remarks

Cyanobacteria possess a strong potential as actors in the biotechnology sector owing to their long natural evolution, dependence on widely available utilities (carbon dioxide and sunlight), ease of cultivation, and versatile metabolism. However, the performance of photobioreactors for their growth has lagged far behind that of classical fermenters. Of particular concern is the efficiency of light and gas transfer, yet advantages in downstream processing are needed as well.

Optimization of processing conditions must be done consistently, and the unit costs of harvesting must be considerably decreased. Moreover, an integrated approach to the whole process is demanded, based on a biorefinery strategy in which the various metabolites synthesized are recovered and any residues/effluents are turned into usable products. This not only minimizes environmental impacts but also enhances the economics underlying the process, hitherto characterized by poor volumetric efficiencies.

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Chapter 18

Commercial-scale culturing of cyanobacteria: an industrial experience

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18.1 Introduction

Worldwide, cyanobacteria (mainly *Spirulina* and *Nostoc*) are being commercially cultivated for food and feed. Different cultivation systems such as open ponds and closed photobioreactors are currently being used for commercial large-scale cultivation of phototrophs (cyanobacteria and other microalgae) (Zhang, Miyachi, and Kurano, 2001; Chisti, 2007; Lehr and Posten, 2009; Posten, 2009; James and Boriah, 2010). Table 18.1 summarizes the advantages and disadvantages of various culture techniques (Anonymous, 1991).

In the majority of commercial plants, open raceway culture ponds are used for the production of *Spirulina* biomass. Closed photobioreactors are used for the production of *Nostoc* biomass. In the following sections we will provide overviews of open ponds and closed photobioreactors, with reference to the large-scale culturing of *Spirulina* and *Nostoc*.

18.2 Open-pond systems

The large-scale cultivation of cyanobacteria in outdoor open-pond systems is well established. Amongst the different types of open ponds designed, only four have been developed and operated on large scales. These are:

- unmixed ponds such as natural ponds and lakes
- thin layer, inclined systems, where mixing is achieved through pumping and gravity flow
- circular ponds with agitation provided by a rotating arm
- raceway ponds constructed as an endless loop in which the culture is circulated by paddlewheels.

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Table 18.1 Advantages and disadvantages of different culture techniques.

Culture type	Advantages	Disadvantages
Indoors	A high degree of control	Expensive
Outdoors	Cheaper	Little control
Closed	Contamination less likely	Expensive
Open	Cheaper	Contamination more likely
Axenic	Predictable, less prone to crashes	Expensive, difficult
Non-axenic	Cheaper, less difficult	More prone to crashes
Continuous	Efficient, provides a consistent supply of high-quality cells, automation, highest rate of production	Difficult, usually only possible to culture small quantities, complex, expensive
Semi-continuous	Easier, somewhat efficient	Sporadic quality, less reliable
Batch	Easiest, most reliable	Least efficient, quality may be inconsistent

Source: Anonymous, 1991.

The most commonly used ponds for cyanobacteria are raceway ponds.

18.2.1 Raceway ponds

Raceway ponds have been used for mass culture of microalgae since the 1950s, and there is extensive experience of the operation and engineering of raceways. A raceway pond is made up of a closed-loop recirculation channel (Figure 18.1). Raceway channels are constructed of concrete or compacted earth, covered with white, blue, or black plastic sheets. Mixing and circulation are brought about by a paddlewheel. The paddlewheel operates continuously to prevent sedimentation of the cells. Low building and operating costs are the biggest advantages of raceway ponds. The cost of building a raceway pond is strongly influenced by the characteristics of the ground on which it is built. The lining must be fixed to the ground in order to avoid displacement by wind. However, raceway ponds have many drawbacks. Large raceway ponds cannot be operated at depths greater than 25 cm; otherwise a severe decrease of flow and turbulence will occur. The depth of raceway ponds is usually maintained at 15–25 cm because of the attenuation of the sunlight at greater depths. At night, a decrease in the biomass content is usually observed.

In raceway ponds cooling is achieved by evaporation, which is affected by ambient temperature. However, temperature fluctuates during the diurnal cycle and also seasonally. In hot and dry climates evaporation losses may be excessive and therefore carbon dioxide is not used effectively in raceway ponds. Productivity in the open raceway ponds is also affected by contamination by unwanted algal species and other microorganisms. Raceway ponds are therefore only suitable for a small number of phototrophic microorganisms that

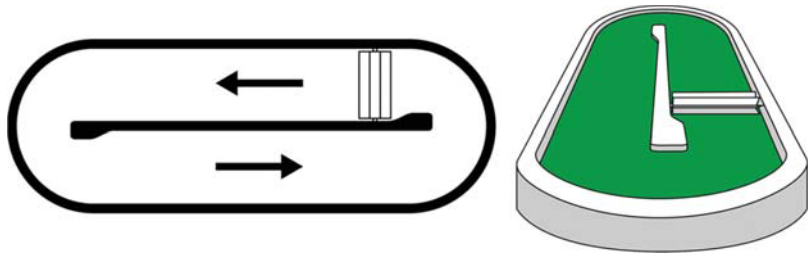

Figure 18.1 Schematic diagram of a raceway pond. See plate section for color version.

tolerate extreme environmental conditions and which can therefore outcompete other species.

18.3 Closed photobioreactors

Closed photobioreactors were developed to overcome the problems associated with open-pond systems. These include contamination, uncontrollable environments, evaporation, low productivity, and the need for large land areas. Closed photobioreactors can be located indoors and be provided with artificial light or natural light via light collection and distribution systems, or be located outdoors to use sunlight directly. However, closed photobioreactors also have certain disadvantages. For example, they are more expensive and more difficult to scale up than open ponds. Indoor closed photobioreactors involve complex and costly light collection and distribution systems. Moreover, light limitations cannot be entirely overcome since light penetration is inversely proportional to cell density. Wall growth of cells may also decrease the light inside the reactor.

Many different types of photobioreactors have been designed, but only three have been developed and operated at large scales:

- tubular photobioreactors
- column photobioreactors
- flat-plate photobioreactors.

18.3.1 Tubular photobioreactors

The most widely used closed photobioreactors are the tubular type. The tubes are generally less than 10 cm in diameter in order to maximize light penetration. The culture is circulated through the tubes by a pump, exposing the cyanobacterium (or other algae) in the nutritional growth medium to light, encouraging photosynthetic growth. In a typical arrangement the tubes are placed parallel to each other lying flat above the ground (Figure 18.2a). Straight horizontal tubes are arranged in parallel, like a fence (Figure 18.2b). Instead of being laid horizontally on the ground, the tubes may be made of flexible plastic and coiled around a supporting frame to form a helical coil tubular photobioreactor (Figure 18.2c).

18.3.2 Column photobioreactors

Column photobioreactors (also known as cylindrical photobioreactors) are simple systems in which mixing is achieved by injecting compressed air from the bottom. A disposable vertical reactor can be easily built by cutting a suitable length of transparent polyethylene tubing heat-sealed at one end. This reactor can be suspended from a framework or supported within a mesh frame (Figure 18.3a). However, such reactors have a short lifetime because of bio-fouling, but are easy and inexpensive to replace. Rigid vertical columns, typically 1–2.5 m in height and 30–50 cm in diameter are also used (Figure 18.3b). These reactors are used

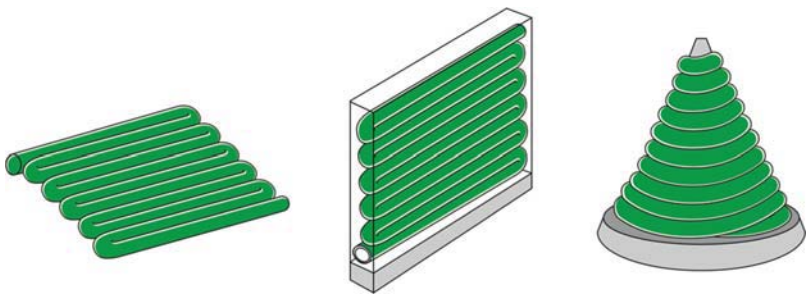

Figure 18.2 Tubular photobioreactors. The tubes can be placed parallel to each other lying flat above the ground (a), horizontal tubes can be arranged in parallel (b), or tubes made up of flexible plastic can be coiled around a supporting frame to form a helical-coil tubular photobioreactor (c). See plate section for color version.

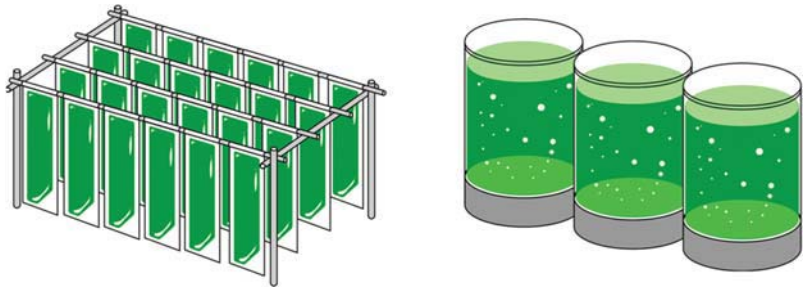

Figure 18.3 A column photobioreactor. Column photobioreactors can be suspended from a framework or supported within a mesh frame (a), or they can be rigid vertical columns (b). See plate section for color version.

indoors with artificial illumination, in greenhouses with natural light, or outdoors. In outdoor systems, cooling is by water spraying.

18.3.3 Flat-panel photobioreactors

Flat-panel photobioreactors are made of thin rectangular transparent boxes for maximum utilization of light (Figure 18.4). Aeration and mixing are provided via a perforated tube running along the bottom of the reactors. Despite their apparent simplicity, few such systems have been used for mass cultivation (Tredici, 1999).

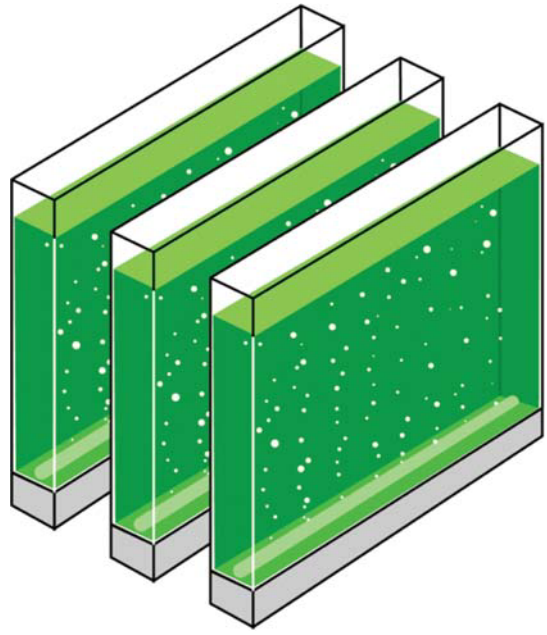

Figure 18.4 A flat panel photobioreactor. See plate section for color version.

18.4 Comparison of open raceways and closed photobioreactors

As set out in Table 18.2, the main advantages of raceway ponds are their ease of operation and maintenance, resulting from the simple design and low energy requirement. Closed photobioreactors have high productivity, low risk of contamination, and are suitable for different species of cyanobacteria.

Various authors have estimated the cost of producing biomass in raceway ponds and in closed photobioreactors (Table 18.3). The major cost of a raceway pond is associated with its operation (labor, utilities, and raw materials). In contrast, the cost of production in photobioreactors is dominated by capital costs. Therefore, producing biomass in photobioreactors is more expensive than in raceway ponds. Selecting a suitable method for cyanobacterial production requires a comparison of the capabilities of raceway ponds and photobioreactors.

Table 18.2 Comparison of raceway ponds and photobioreactors.

Factor	Raceway ponds	Photobioreactors
Cell density in culture	Low	High
Culture volume necessary to harvest a unit weight of cells	High	Low
Commercial availability	Readily available	Usually custom built
Construction costs per unit volume produced	Low	High
Operating costs	Low	High
Technology base	Readily available	Under development
Risk of contamination	High	Low
Weather dependence	High	Low
Maintenance	Easy to maintain	Difficult to maintain
Susceptibility to overheating	Low	High
Susceptibility to excessive oxygen levels	Low	High
Ease of cleaning	Easy	Difficult
Ease of scale up	Easy	Variable
Land requirement	High	Variable
Applicability to different species	Low	High

Modified from Alabi, 2009 and Brennan and Owende, 2010.

Table 18.3 The estimated costs of cyanobacterial biomass production.

	Cost/kg of dry biomass (€)	
	Raceway ponds	**Closed photobioreactors**
Alabi (2009)	2.00	5.63
Norsker *et al.* (2011)	4.95	4.15 (tubular photobioreactor) 5.96 (flat-panel photobioreactor)
AquaFUELs (2011)	0.4–1.8	4–10

Figures given for Alabi, 2009 are converted from US dollars at a rate of $1 = €0.77.

18.5 *Spirulina*

Spirulina (*Arthrospira)* is a free-living, planktonic filamentous cyanobacterium characterized by a cylindrical, multicellular trichome, with an open left-hand helix (Ciferri, 1983). It is edible, with nutritional as well as potential therapeutic values (Khan, Bhadouria, and Bisen, 2005; Karkos *et al.*, 2011). In nature, *Spirulina* (*Arthrospira)* grows in high-salt alkaline reservoirs in subtropical and tropical areas in America, Asia, and Central Africa (Habib *et al.*, 2008). Some of the commonly known species include *S. platensis*, *S. maxima*, and *S. fusiformis*.

As early as 400 years ago, during the Aztec civilization in Mexico, the Mayas, Toltecs, and Kanembu ate *Spirulina* as part of their diet (Ciferri and Tiboni, 1985). *Spirulina* growing in Lake Texcoco were harvested, dried, and used to make a cake. The Chadians in Central Africa also consume *Spirulina*. *Spirulina* collected from Lake Kossorom (Chad) has been used to make cakes or broths for consumption (Abdulqader, Barsant, and Tredici, 2000). The nutritional value of *Spirulina* is well recognized. This organism has a high protein content (60–71% of dry weight) and is rich in vitamins, minerals, essential fatty acids, and other nutrients (Dillon, Phuc, and Dubacq, 1995) (see Chapter 7 for details on *Spirulina*). The use of *Spirulina* has expanded from its original application as food – where its main use being as a neutraceutical and functional food supplement – and feed to the production of fine chemicals for clinical, biological, and cosmetic applications (Deng and Chow, 2010).

The commercial large-scale culture of *Spirulina* started in the early 1970s at Lake Texcoco, Mexico (Borowitzka, 1999). Among the different species of edible *Spirulina*, *S. platensis* was the first to be commercially cultivated. Today, *Spirulina* is produced in at least 23 countries: Benin, Brazil, Burkina Faso, Chad, Chile, China, Costa Rica, Cote d'lvoire, Cuba, Ecuador, France, India, Japan, Madagascar, Mexico, Myanmar, Peru, Israel, Spain, Thailand, Tog, the United States of America, and Vietnam.

Commercial-scale cultivation of *Spirulina* is carried out in open-raceway ponds equipped with paddlewheels. Figure 18.5 shows a raceway pond on Miyako Island, Japan. The following is a brief description of the processes and some of the problems associated with the large-scale culture of *Spirulina*.

18.5.1 Cultivation system

The surface of raceways varies from 300 to 5000 m^2. Mixing is facilitated by means of a single or double paddlewheel. The depth of the culture (usually maintained at 15–25 cm) depends on the season, desired algal density, and to a certain extent on the desired biochemical composition, particularly that of pigment content. Experiences from many *Spirulina* production facilities suggest that the paddlewheel mixing does not provide sufficient turbulence to maintain the cyanobacteria at the optimum residence time in the photic zone. Recommended flow velocities of the order of 60 cm/s have been reported (Richmond, 1988). However, in our experience increasing the flow velocity above 30 cm/s invariably results in fragmentation, decreased yield, and in increased expenditure on pond maintenance and energy. The optimum flow velocity for high growth rates depends on the operational depth of the raceway pond, the algal density, and the physiological state of the *Spirulina*. Therefore, we recommend a flow rate of 15–30 cm/s.

Figure 18.5 A raceway pond in Miyako Island, Japan. See plate section for color version.

18.5.1.1 Culture medium

The culture medium contains sodium carbonate, nitrogen, phosphorus, iron, and trace metals. After harvesting of the biomass, the culture medium is recycled and returned to the ponds throughout the growth season. The nature and concentration of the nutrients used is a matter of cost and compatibility with seawater. Every production facility has its own, often secret, approach to obtaining optimum growth rates and yields of *Spirulina* at minimum cost.

A major problem encountered during outdoor culturing of *Spirulina* is the high calcium content of seawater. Calcium carbonate crystals precipitate during cultivation, resulting in loss of the alkalinity of the medium.

18.5.1.2 Temperature

Temperature is a main limitation for high biomass production of the *Spirulina* culture in outdoor ponds. The optimal temperature for cultivation of *Spirulina* is in range of 35–38°C (Habib *et al.*, 2008). It has been observed that high temperatures at night increase the rate of respiration, causing loss of biomass.

18.5.1.3 Contamination

Contamination by different microalgae and/or other microorganisms is a severe problem for the cultivation of *Spirulina* in outdoor open ponds. The maintenance of cultures consisting only of *Spirulina* is a challenge to any commercial culture facility. The constant recycling of the medium results in excessive accumulation of organic matter. Contamination by other microorganisms may result in the inhibition of growth of *Spirulina*. The accumulation of organic matter in the medium also causes, at times, excessive foaming, due to the

death and decomposition of *Spirulina*. Contamination by zooplankton and/or protozoans also occurs. Predation of *Spirulina* by large ciliates has been observed.

18.5.1.4 Harvesting

Spirulina biomass is harvested from culture ponds by means of filtration. Three types of filtration device are available:

- an inclined gravity screen
- a horizontal vibratory screen
- a vacuum-table or vacuum-belt filter.

It has been suggested that operation of all three devices in series may improve harvesting capacity and efficiency, as well as causing less damage to the organism. The process provides a suspension of 8–15% of dry weight.

18.5.1.5 Drying

There are many types of drying systems used by *Spirulina* facilities. Thorough and quick drying provides high quality *Spirulina*. It is essential to have the drying process completed in minutes in order to ensure preservation of heat-sensitive compounds such as pigments or certain enzymes. The drying approaches commonly recommended are either spray-drying or drum-drying systems. Despite its high cost the spray-drying system is preferred over the drum-drying system.

18.5.1.6 Packaging

Proper packaging of high-quality *Spirulina* powder is important for its marketing purposes. *Spirulina* powder coming out of the drier is immediately sealed under a vacuum in an oxygen barrier bag with a deoxidizer. This minimizes oxidation of certain vital pigments such as carotenoids.

18.6 *Nostoc*

Nostoc is a genus of filamentous cyanobacteria that forms macroscopic or microscopic colonies and is common in both terrestrial and aquatic habitats. Moreover, *Nostoc* has been reported from both the North and the South Poles (Potts, 2000). It is found in gelatinous colonies and is composed of trichomes surrounded by a thin sheath (Mollenhauer, 1988).

Nostoc is an ancient taxon. Fossils recovered from shallow marine sediments from the late Precambrian are morphologically similar to modern forms (Dodds, Gudder, and Mollenhauer, 1995). *Nostoc* trichomes possess differentiated specialized cells (heterocysts), which are the site of nitrogen fixation, and hence these organisms are important to maintain soil fertility in many terrestrial systems, including rice paddies.

Nostoc is both useful and a nuisance to humans. *N. commune*, *N. flagelliforme*, and *N. sphaericum* are regionally (Asia and South America) popular edible delicacies (Figure 18.6). Different studies have reported their hypocholesterolemic, anti-bacterial, anti-viral, anti-infectious, anti-oxidant, and immune-enhancing activities (Hori, Ishibashi, and Okita, 1994; Jaki, Heilmann, and Sticher, 2000; Kanekiyo *et al.*, 2007). The State Council of the People's Republic of China prohibited the collection and trading of *N. flagelliforme* in June 2000 in an attempt to stop desertification, which resulted from overexploitation of this organism in order to satisfy increasing market demand in northern China (But *et al.*, 2002).

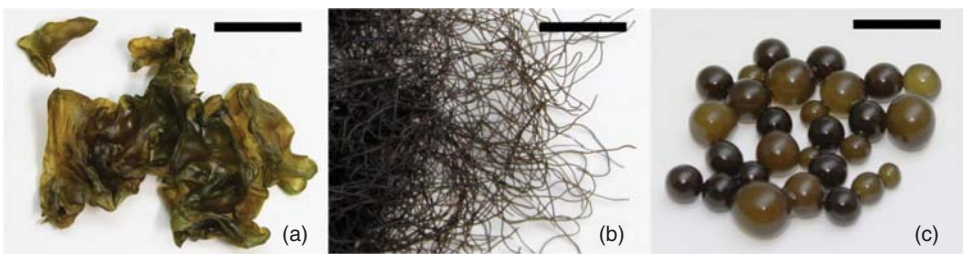

Figure 18.6 (a) *N. commune*; (b) *N. flagelliforme*; (c) *N. sphaericum* (scale bar = 1 cm). See plate section for color version.

The large-scale culture of certain species of *Nostoc* is well established in Japan and China; *N. commune* and *N. flagelliforme* have been used as herbal ingredients and have also been a food delicacy for hundreds of years. *N. commune* and *N. flagelliforme* are terrestrial species and grow slowly on solid media. This is inappropriate for commercial production and therefore, *N. commune* and *N. flagelliforme* are grown in liquid media in photobioreactors, which vary in volume from 100 to 1000 l. Figure 18.7 shows a 200-l column photobioreactor. Free-living cells isolated from natural colonies of *N. commune* and *N. flagelliforme* are used for large-scale cultivation. Bubbling with air, with or without additional carbon dioxide, brings about mixing, but sometimes a propeller used as well. The culture medium contains sodium carbonate, nitrogen, phosphorus, iron, and trace metals. The precise composition of the medium for both *Nostoc* species is secret. The optimal temperature for cultivation of *Nostoc* is in the range of 20–30°C. Harvesting, drying, and packaging are similar to the methods described above for *Spirulina*.

N. sphaericum grows naturally during the winter in mountain paddy fields in China. It forms macroscopically visible spherical colonies (Qiu *et al*., 2002). It also grows in the high-altitude Peruvian sierra (Nunez and Mendoza, 2006). In China, *N. sphaericum* has been used as a food delicacy and as an ingredient of Chinese medicine since the Eastern Jin Dynasty (317–420 AD).

Figure 18.7 A 200-l column photobioreactor. See plate section for color version.

In China the commercial culture of *N. sphaericum* started in 2008.

N. sphaericum is cultivated in liquid media in column reactors, which vary in size from 50 to 200 l. Mixing is achieved by bubbling, with or without carbon dioxide added. The cyanobacterium may be grown in BG-11 but in this medium the growth rate is slow. Each commercial facility uses its own secret formulation for growing *N. sphaericum*. The optimal temperature for cultivation is in the range 15–20°C. It is easy to harvest this cyanobacterium using a net.

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Chapter 19

Engineering cyanobacteria for industrial products

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19.1 Introduction

Cyanobacteria have been used commercially for many years as a source of diverse naturally produced compounds, as described in other chapters of this volume. As genetic tools can easily modify several cyanobacterial genera, development of these techniques now allows the introduction of foreign genes, enabling us to establish production of valuable new products and to improve of the production of natural compounds.

This article focuses on a discussion of the tools available for genetic engineering of cyanobacteria. Most of the systems currently used to genetically manipulate cyanobacteria can be traced back to the 1970s and 1980s, when the first unicellular and filamentous strains were engineered to perform mutational analyses (Table 19.1). Since the time of these early studies, there have been very important new developments, namely the extensive sequencing of the genomes of different cyanobacterial strains (e.g., Shih *et al.*, 2013; Stanley, Raines, and Kerfeld, 2013) and new approaches in transcriptome analysis (Mitschke *et al.*, 2011a, b). Therefore, the potential of genetic engineering in cyanobacteria is nowadays widely acknowledged (Ducat, Way, and Silver, 2011). However, commercial exploitation of this potential is still in its infancy. The current focus for commercialization of engineered cyanobacteria is their use as a sustainable source of biofuels and chemical feedstocks.

19.2 Genetic manipulation of cyanobacteria

19.2.1 Transformation and electroporation

Several cyanobacterial strains, including the first genetically engineered strains, are naturally transformable (Table 19.1). Exogenous DNA is taken up by these strains without any pretreatment of the cells. For these

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Table 19.1 Genetic manipulation strategies for cyanobacterial strains.

Strain	Type of manipulation	Reference
Unicellular strains		
Chroococcidiopsis sp.	Conjugation Electroporation	Billi *et al.*, 2001
Synechococcus sp. PCC 6301	Conjugation	Tsinoremas *et al.*, 1994
Synechococcus sp. PCC 7002 (*Agmenellum quadruplicatum* PR-6)	Transformation	Stevens and Porter, 1980
Synechococcus sp. WH7803, WH8102, WH8103	Conjugation	Brahamsha, 1996
Synechococcus elongatus PCC 7942 (*Anacystis nidulans* R2)	Transformation	Golden and Sherman, 1984
Synechococcus PCC 7943 (*Anacystis nidulans* strain 602)	Transformation	Shestakov and Khyen, 1970
Synechocystis sp. PCC 6714	Electroporation	Ludwig *et al.*, 2008
Synechocystis sp. PCC 6803	Transformation Conjugation	Grigorieva and Shestakov, 1982; Kreps *et al.*, 1990
Multicellular strains		
Anabaena sp. M131	Electroporation	Thiel and Poo, 1989
Anabaena variabilis ATCC 29413	Conjugation	Maldener *et al.*, 1991; Murry and Wolk, 1991
Calothrix sp. PCC7601 (*Fremyella diplosiphon*)	Electroporation	Chiang, Schaefer, and Grossman, 1992
Chlorogloeopsis fritschii PCC 6912	Conjugation	Stucken *et al.*, 2012
Fischerella muscicola PCC 7414	Electroporation Conjugation	Stucken *et al.*, 2012
Nostoc sp. PCC 7120 (*Anabaena* sp. PCC 7120)	Electroporation Conjugation	Chaurasia, Parasnis and Apte, 2008; Wolk *et al.*, 1984
Nostoc sp. PCC 7121	Electroporation	Moser, Zarka, and Kallas, 1993
Leptolyngbya sp. BL0902	Conjugation	Taton *et al.*, 2012
Limnothrix (*Pseudanabaena* sp. ABRG5-3)	Conjugation	Asayama, 2012
Plectonema boryanum PCC 73110	Conjugation	Vachhani, Iyer, and Tuli, 1993
Pseudanabaena NKBG040605C	Conjugation	Sode *et al.*, 1992
Spirulina platensis	Electroporation	Toyomizu *et al.*, 2001
Oscillatoria MKU 277	Electroporation	Ravindran, Suguna, and Shanmugasundaram, 2006

Alternative names of the respective cyanobacterial strain are given in parentheses.

purposes suicide vectors, which do not replicate in the recipient cell, are usually used. The stable maintenance of the recombinant DNA is allowed by integrating it into the cyanobacterial genome. Usually, these strains show efficient homologous double recombination between regions of identical sequence that flank the foreign DNA sequence on both sites. In addition, integration via transposition is possible (Cohen *et al.*, 1994; Leganes, Fernandez-Pinas, and Wolk, 1998; Okamoto, Ikeuchi, and Ohmori, 1999). In *Synechocystis* sp. PCC 6803, DNA uptake of double-stranded DNA involves its conversion to single-stranded molecules (Barten and Lill, 1995), preventing the introduction of self-replicating plasmids in their replicable form into this cyanobacterium by transformation. This can be achieved, however, by electroporation or conjugation (see below). Why only a few unicellular strains are able to uptake exogenous DNA

naturally is not clear. It is known that type IV pili are important structures for DNA uptake, not only in cyanobacteria (Lorenz and Wackernagel, 1994, Yoshihara *et al.*, 2001). Thus, cyanobacteria lacking these appendages might not be able to absorb and transport DNA molecules into the cell. Another reason may be extracellular nucleases or degradation of transferred DNA by the restriction-modification system of the host. Recently, another bacterial immune defense system was discovered, the clustered regularly interspaced short palindromic repeat (CRISPR) system. Similar to RNA interference in eukaryotes, CRISPR-mediated adaptive immune systems rely on small RNAs for silencing foreign nucleic acids, including bacteriophage nucleic acids and plasmids (reviewed recently in Wiedenheft, Sternberg, and Doudna, 2012). For example, Marraffini and Sontheimer (2008) showed that CRISPR interference prevents conjugation and plasmid transformation in *S. epidermidis*. Including new spacers in their CRISPR loci that are homologous to invading DNA, bacteria may acquire immunity against phages or plasmids and pass it on to their progeny. Many cyanobacteria encode CRISPR systems (Scholz *et al.*, 2013; Shih *et al.*, 2013) but their function as immune systems has not been shown so far.

19.2.2 Conjugation

A more general method to transfer DNA to cyanobacterial cells is conjugation. Usually the broad-host range vector RP4 is used to mobilize plasmids that harbor the *oriT* region, based on the RSF1010 plasmid or the *bom* site of pBR321. Shuttle vectors that replicate in *E. coli* as well as in cyanobacteria are useful tools for the expression of foreign genes. Such vectors are the RSF1010-based plasmids that belong to the IncQ group of plasmids (for detailed information on the mechanism and host range see Thiel and Poo, 1989, Zinchenko *et al.*, 1999, and Huang *et al.*, 2010). If plasmids are used that can be transferred using conjugation but do not replicate in the cyanobacterial cell, the DNA fragments have to integrate into the genome to facilitate stable maintenance of the genetic information. To be integrated into the chromosome the vector has to harbor homologous DNA fragments. During a single recombination event the plasmid DNA is eventually integrated into the genome of the recipient. If the aim of the mutation is gene replacement, a double recombination event should occur. In some cyanobacteria, such as *Synechocystis* sp. PCC 6803, usually only double recombination events are detectable. When using other strains (e.g., *Nostoc* sp. PCC 7120) one has to select for the second recombination event. This could be more efficiently done under positive selection pressure using the levan sucrase gene (*sacB*) from *Bacillus subtilis* (Cai Y.P. and Wolk, 1990). This system is used also for many other bacterial species, such as *Rhodobacter sphaeroides* (Suwanto and Kaplan, 1992) or *Myxococcus xanthus* (Wu S.S. and Kaiser, 1996). When *sacB* is expressed, sucrose is lethal for these bacteria. If the *sacB* gene is lost during a double cross-over event, cells can survive on plates supplemented with sucrose.

Many cyanobacteria harbor efficient restriction-modification systems. Thus, several researchers have improved the conjugation system, especially for *Nostoc* sp. PCC 7120, by using a helper plasmid that encodes methylases to modify incoming DNA (Elhai *et al.*, 1997). Another option is the deletion of all sites for the restriction endonucleases of the host strain in the conjugative vector (Wolk *et al.*, 1984).

19.3 Engineering cyanobacteria to express genes of interest

Efficient expression systems are critical for the development of a specific strain into a production strain. An optimal expression system should have several characteristics, which will be described in the following subsections.

19.3.1 Promoters

The promoter should be strong enough to facilitate sufficient mRNA accumulation. Several laboratories use the strong light-induced *psbA2* promoter for expression (Hübschmann *et al.*, 2001; Lindberg *et al.*, 2010). However, for production of toxic compounds a promoter should be used that assures repression of transcription until the production process starts.

This strategy firstly ensures optimal growth of the starting culture and secondly reduces accumulation of mutations in response to the produced compounds that might lower production efficiency. Isopropyl-β-D-thiogalactosid is widely used in *E. coli* expression systems to induce transcription. In industrial production, especially of low-value products, addition of costly inducers is not possible. Several laboratories use the copper-responsive promoters *petE* or *petJ* for controllable expression in cyanobacteria (Zhang *et al.*, 1992; Tous, Vega-Palas, and Vioque, 2001; Kuchmina *et al.*, 2012). The *petE* gene encodes the small water-soluble copper-protein plastocyanin. The alternative electron carrier to photosystem I, the cytochrome c_{553}, is encoded by the *petJ* gene. The transcription of these two genes is regulated by the availability of copper in the growth medium. Zhang *et al.* (1992) showed that cells grown in medium with 0.3 µM copper sulfate (normal BG-11) contain both proteins. Medium containing 1 µM copper sulfate represses the expression of c_{553} to undetectable levels and plastocyanin is present, whereas in cells grown under copper limitation cytochrome c_{553} is mainly expressed. The regulators of these promoters are not known so far. For some applications a high gene expression requires the use of higher copy-number plasmids. In these cases the quantity of the unknown copper regulator might be too low to ensure full repression. Another useful controllable system represents the *ziaAR* operon. Here, ZiaR is a Zn^{2+}-responsive repressor of *ziaA* transcription, a gene encoding a heavy metal transporting P-type ATPase (Thelwell, Robinson, and Turner-Cavet, 1998). Transcription from the *ziaA* promoter is greatly increased by the addition of 2 µM Zn^{2+} and repressed in the absence of the metal ion.

19.3.2 Gene copy number

Many cyanobacteria harbor more than one chromosome per cell, and the number may change under different growth conditions (Griese, Lange and Soppa, 2011). Introduction of a gene into the chromosome may therefore lead to several additional gene copies. Self-replication plasmids may have a higher copy number and therefore are better suited for maximal expression of a metabolic enzyme. For overexpression of genes in *Synechococcus* sp. PCC 7002 the endogenous plasmid pAQ1 is used (Xu *et al.*, 2011). This smallest plasmid of the cyanobacterium, of 4809 bp, has a copy number of around 50 per cell, which is about eight times more than the copy number of the chromosome. There exist gene expression cassettes that are linked to plasmid-derived flanking regions for integration of these overexpression cassettes into a neutral site of the plasmid. Inserts of up to 14 kbp have been successfully introduced into the pAQ1 plasmid (Xu *et al.*, 2011).

19.3.3 mRNA stability and post-transcriptional regulation

Transcript stability is often ignored in attempts to produce large amounts of protein. The accumulation of a specific mRNA does not solely depend on the promoter used for expression of a gene. The cellular concentration of a transcript depends both on the rates of its synthesis and degradation and thus the level of a protein is controlled by transcription and RNA degradation. mRNA decay is highly regulated in bacteria and involves activity of RNases, polyadenylation, ribosome binding, and post-transcriptional regulation by small RNAs. In order to stabilize a transcript, terminator structures and stabilizing elements at the 3′ end of an RNA are important. Hienonen *et al.* (2007) showed that the 5′ and 3′ elements of an extremely stable mRNA of *Pseudomonas syringae* can confer stability to mRNAs encoding heterologous proteins, and also increase production of the heterologous protein. In practice, to enhance mRNA accumulation in the cell either the own 3′ UTR of an mRNA should be used or a rho-independent terminator has to be fused to the 3′ end of the gene sequence. The stabilizing effect of ribosome binding on heterologous mRNA has also to be considered when rare codons are removed, in order to adjust codon usage. Rare codons might enhance the ribosome protection effect on mRNA stability by changing the secondary structures of transcript (Wu *et al.*, 2004).

19.3.4 Antibiotic-resistance-free systems

Since the intended genetic events happen extremely seldom it is absolutely necessary to link a selection pressure to the introduced mutation. In order to

identify cells that maintain the new genetic material, antibiotic or herbicide resistance cassettes are usually introduced alongside the respective mutated gene. However, for the use of genetically engineered organisms in industrial production, antibiotics and antibiotic-resistant cells are usually forbidden by regulatory authorities. In addition, when creating multiple mutations one might be restricted by the number of available resistance-gene cassettes. There are several such selection markers available for different strains (for review see Ruffing, 2011). However, for production strains economic, low-cost, and non-toxic solutions are needed and markerless mutants have therefore been developed for several bacteria. In cyanobacteria, two systems are mainly used: the *sacB*- and *rps12*-related systems. Both are gene-replacement techniques that introduce a specific sensitivity combined with a common antibiotic-resistance gene cassette in a first step. The antibiotic resistance drives the selection pressure for the first step. In a second step the sensitivity gene, including the antibiotic-resistance gene cassette, is replaced by a gene of interest and selection is driven by loss of the sensitivity (Clerico, Ditty, and Golden, 2007). *rps12*-mediated gene replacement is based on the fact that several point mutations in the *rps12* gene, which encodes the ribosomal protein S12, lead to streptomycin resistance in cells harboring the mutated gene version. First, the *rps12* gene has to be replaced by a mutated version using homologous recombination, thus creating a streptomycin-resistant strain. Then the sensitivity gene (in this case the wild-type *rps12* gene) in combination with an antibiotic resistance gene will be used to replace a gene of interest. In a third step both the sensitivity and the antibiotic-resistance genes are replaced by homologous recombination using the mutated gene of interest linked to the streptomycin-resistance-conferring mutant *rps12* gene. Transformants can now be selected on streptomycin resistance. When using the *sacB* gene, a procedure including the last two steps will lead to cells that can be selected because of their ability to grow on sucrose. Both systems are described in detail by Cai and Wolk (1990), Matsuoka *et al.* (2001), and Ruffing (2011). There are two main problems when using these strategies. First, the possibility to select further for the mutation is lost. This could be problematic if expressing potentially toxic gene products or metabolic pathways that convert fixed carbon away from biomass formation towards a product of interest (for example enzymes for biofuel production). Production strains, especially if the gene products are encoded on a plasmid, will quite frequently lose their ability to produce such compounds. Second, the procedures are very time consuming, especially when using strains with high genome copy numbers such as *Synechocystis* sp. PCC 6803. Here, it is highly important to replace all wild-type alleles with the sensitivity marker. If this is not the case the subsequent selections of transformants on streptomycin resistance or sucrose tolerance will fail, because selection of the remaining unmodified alleles will be likely.

We suggest as an alternative the establishment of an antibiotic-free expression system for generation of production strains (see Figure 19.1). This would be based on auxotrophy. The first step is the construction of a cyanobacterial auxotrophic mutant (e.g., histidine auxotrophy). The *hisB* gene (in other species called *his3*) encodes the imidazole glycerol-phosphate dehydratase, an essential enzyme of the histidine biosynthesis pathway. Inactivation of the *hisB* gene results in a histidine auxotrophic mutant. This auxotrophy marker is commonly used in yeast genetics but also in *E. coli*. In order to create a cyanobacterial histidine auxotrophic mutant, the *hisB* gene (in *Synechocystis* sp. PCC 6803, *slr0500*) has to be knocked out by a complete deletion of *hisB*, using the *sacB* system for markerless deletion. Now the genes of interest (e.g., for biofuel production) will be introduced on a self-replicating plasmid also harboring the *hisB* gene, thus complementing the histidine auxotrophy. The *hisB* gene cassette can also be used for introduction of genes or to make deletions in the chromosome, when the histidine auxotrophic strain will be used as recipient.

Recently, another method for markerless genetic modification has been demonstrated for *Synechocystis* sp. PCC 6803 (Cheah, Albers, and Peebles, 2013). The authors used the toxic *mazF* gene (a part of a toxin/antitoxin system) encoding a sequence-specific endoribonuclease as a counter-selection marker, similar to the *sacB* selection method.

19.3.5 Mutagenesis

Generation of genetically engineered cyanobacteria may be a common step for the selection of suitable

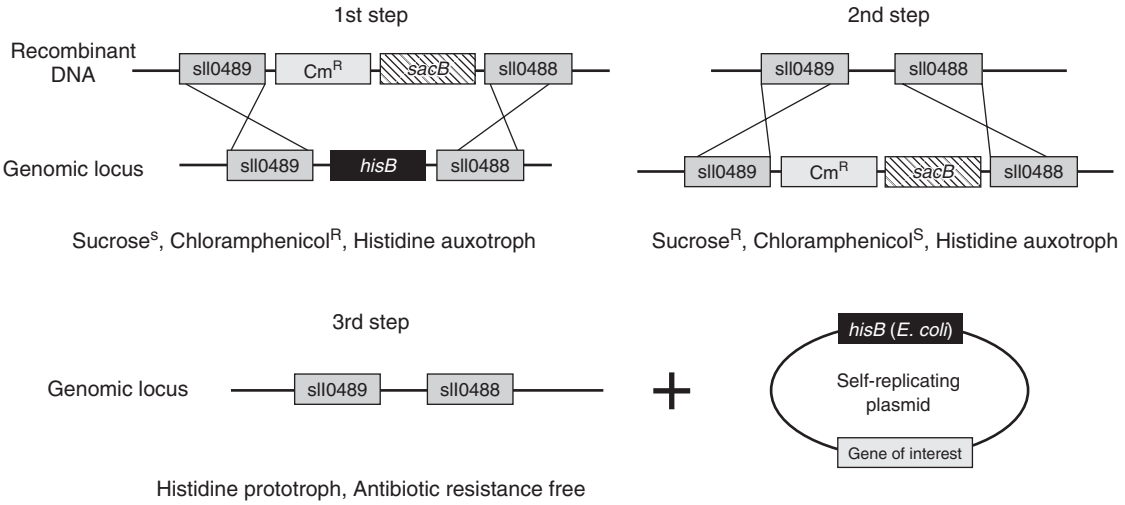

Figure 19.1 Scheme for the use of auxotrophic mutants to construct an antibiotic resistance gene free expression mutant. The first step is the generation of a sucrose sensitive histidine auxotroph. Mutants are selected due to insertion of an antibiotic resistance gene cassette (e.g., chloramphenicol resistance). With the second step the antibiotic resistance gene cassette is removed and transformants are selected on sucrose. In the last step an expression plasmid harboring the gene of interest fused to a strong promoter is transferred to the mutants followed by selection on histidine prototrophy.

producer strains. In recent years many different approaches have been developed to generate specific mutants. These approaches include single site-directed mutagenesis (mainly for the study of specific protein functions), deletion mutagenesis of a specific gene, random mutagenesis, introduction of new heterologous pathways, and overexpression of genes. The modes of mutagenesis in cyanobacteria and their applications have been thoroughly and excellently reviewed by Koksharova and Wolk (2002) and Ruffing (2011). In this review we will focus on the discussion of methods that have been used in other bacteria to select optimized producer strains through directed-evolution approaches.

The rational and intuitive approaches to engineering bacteria to produce chemicals via non-native metabolic pathways are often insufficient to maximize production or to optimize overall performance of the cell during the production process. Cellular networks are extremely complex and include, besides the enzymes needed to construct a new metabolic pathway, production of cofactors, transport mechanisms, stress responses, precursor enrichment, and elimination of toxic by-products. System-wide studies, such as DNA-microarray analysis, metabolic profiling, and computer-based optimization of metabolic pathways, represent possible approaches to identify bottlenecks and assigning targets for directed engineering. Another approach to strain improvement is adaptive laboratory evolution. This method is based on natural selection of beneficial mutations and has the advantage that non-intuitive and multiple mutations can be selected. Using this approach, metabolically engineered strains can be selected for improved fitness, higher product yield, or utilization of new substrates. Most of these applications in metabolic engineering have used model organisms such as *E. coli* or yeast. For example, Hu and Wood (2010) used adaptive evolution combined with chemical mutagenesis to select a strain that produced 20 times more hydrogen and 5 times more ethanol than the unimproved strain in medium based on glycerol, a poor energy source. Microarray analysis revealed repression of several pathways that would decrease hydrogen yields, while a beneficial pathway that encoded enolase was induced (Hu and Wood, 2010). Such approaches are especially useful in identifying beneficial mutations that are not easily predictable. Examples include mutations in regulatory elements such as the stationary-phase sigma factor (Herring *et al.*, 2006) or the RNA polymerase subunit

β′ (Conrad *et al.*, 2010). Expression of alien metabolic pathways often leads to a reduced fitness of the cells, and so the cells are not able to grow well under stress conditions or nutrient limitation. In this case, cells should be adapted to the production environment by gradually increasing the stress. Production of ethanol, butanol, or isobutanol in cyanobacteria can be harmful for them and can induce several stress responses. By applying adaptive evolution, production strains with higher tolerance can be selected. For example, Atsumi *et al.* (2010) developed an isobutanol-tolerant *E. coli* strain after 45 rounds of sequential transfers. However, taking into account the much higher doubling times of cyanobacteria, such laboratory evolution experiments will take significantly more time to end up with an optimized production strain. Wang *et al.* (2009) describe a method named multiplex automated genome engineering for evolution of cells. Using this method many locations on the chromosome can be targeted very rapidly in a single cell. The authors applied this approach to optimize the production of lycopene in *E. coli* and within three days were able to isolate mutants with a more than fivefold increase of the product level (Wang *et al.*, 2009).

19.4 Cyanobacteria as cell factories for product syntheses

19.4.1 Requirements of industrial production strains

The natural yields of cyanobacterial products of interest are often rather low. Engineering of cyanobacteria in order to create highly productive strains might help to overcome this limitation of wild-type organisms, and thus to meet productivity expectations. Furthermore, cyanobacteria may well serve as production organisms for substances that do not naturally occur in these organisms. The techniques described in the first two sections of this chapter serve as tools for the introduction of genes into cyanobacteria and the optimization of the expression cassettes as well as the respective production rates. These developments have led to various studies of the use of cyanobacteria as production hosts for a variety of low- and high-value products, and major potential applications will be discussed below.

Compared to other well-established engineerable microorganisms such as *E. coli* or *S. cerivisiae*, as primary producers the photosynthetic cyanobacteria are modest in their cultivation condition requirements: only carbon dioxide, light, water, and some minerals are needed for successful cultivation. This has innate advantages: cyanobacteria do not need a cost- and preprocessing-intensive carbohydrate feedstock, and carbon dioxide capture is an added value for green chemical synthesis. Sunlight can be used as a light source, and many cyanobacterial strains do not require fresh water but are content with brackish or marine water; some even flourish in industrial wastewater. The phototrophic nature of cyanobacteria and the inorganic cultivation media confer significant advantages for process control, including a reduced risk of contaminating microorganisms, which might reduce product yields. Therefore, genetically engineered cyanobacteria are increasingly exploited as a sustainable source of biofuels and chemical feedstocks.

In addition to novel photobioreactor designs ensuring optimal growth, possibilities to optimize productivity include the introduction of biosynthetic enzymes necessary for the production of a target molecule, the optimization of carbon fixation, for example by introducing additional or optimized RuBisCO genes (Iwaki *et al.*, 2006; Parikh *et al.*, 2006; Greene, Whitney, and Matsumura, 2007), or the optimization of light utilization by antenna reductions (Mitra and Melis, 2008, Ort and Melis, 2011).

Further requirements for production cyanobacteria are strain robustness as well as genetic stability to ensure long-term productivity. Increases of stress tolerance against, for example, temperature (Tillich *et al.*, 2012) or salinity (Bagchi *et al.*, 2007; Ruffing, 2011) not only leads to enhanced longevity, but may also serve to protect cyanobacterial cultures against contamination with other (heterotrophic) microorganisms, as happens for, for example, *Arthrospira* mass cultivation.

Also, convenient harvesting of the product is crucial for economic production. If compounds are excreted into the cultivation medium or are volatile (for examples see below), the product harvest can take place during continuous cultivation, without any need

to work up the cell biomass. Intracellular metabolites that cannot yet be produced in an excreted form, on the other hand, require batch production and downstream processing of the harvested biomass to obtain the product, rendering the process less cost effective. The harvesting process can be made more efficient if cells tend to sediment rapidly; this can also be induced by flocculation (Schlesinger *et al.*, 2012). To facilitate this process, self-destruction mechanisms have been introduced into cyanobacteria, causing cell lysis at a defined time point (Liu *et al.*, 2011). Cell walls have also been weakened in general to facilitate diffusion (Liu, Sheng, and Curtiss, 2011).

Last, but not least, for commercial large-scale production using genetically modified organisms, regulatory requirements have to be met to avoid spreading artificial genes or genes conferring antibiotic resistance into the environment. This is especially important when sunlight is to be used as the energy source in outdoor cultivation. In such cases, safety measures against the accidental release of modified organisms must be taken. The development of antibiotic-resistant cassette-free selection systems is of great importance in these cases (Dühring and Lee, 2012).

19.4.2 Low-value products

Low-value products from cyanobacteria comprise on the one hand biofuels (e.g., ethanol, isobutanol, biodiesel, alkanes, hydrogen) and on the other hand chemical feedstocks and raw materials (e.g., isoprene, sugars, lactic acid, amino acids).

19.4.2.1 Biofuels

Due to its immense commercial promise, the production of biofuels using engineered cyanobacteria is one of the most vibrant research areas in this field. As production of biofuels is discussed in Chapter 10 of this volume, we will not go into detail here.

19.4.2.2 Chemical feedstocks and raw materials

Isoprene

Isoprene is mainly used as a precursor of synthetic rubber. It is produced by many plants, but it cannot be readily isolated from them due to its volatility. Isoprene capture in a closed microalgal photobioreactor, however, might be more feasible (Bentley and Melis, 2012), and thus the potential of cyanobacteria to produce isoprene has been studied. Isoprene synthase (IspS) from *Pueraria montana*, converting dimethylallyl pyrophosphate into isoprene, has successfully been expressed in *Synechococcus* sp. PCC 6803, leading to yields of about 50 µg/g dry cell weight/day (Figure 19.2; Lindberg, Park, and Melis, 2010).

Isobutyraldehyde

Isobutyraldehyde is a precursor in the synthesis of chemicals such as isobutanol, isobutyric acid, and neopentyl glycol, which in turn can be used in the synthesis of polyesters, lubricants, and plasticizers. To produce isobutyraldehyde, four enzymes needed to be expressed in *Synechococcus elongatus* PCC 7942: pyruvate is converted into 2-acetolactate by acetolactate synthase (AlsS from *Bacillus subtilis*), which is turned into 2,3-dihydroxy-isovalerate by acetohydroxy acid isomeroreductase (IlvC) and subsequently to 2-ketoisovalerate by dihydroxy-acid dehydratase (IlvD, both from *E. coli*), which finally is converted into isobutyrate by 2-ketoacid decarboxylase (Kdc from *Lactococcus lactis*) in yields of about 6 mg/l/h in a strain with additional enhanced carbon-fixation capabilities due to increased RuBisCO activity (Figure 19.3; Atsumi, Higashide, and Liao, 2009).

Ethylene

Ethylene is one of the most important petrochemical feedstocks and can be used to produce a multitude of products, such as plastics (e.g., polyethylene, polystyrene, or PVC), textile polyesters, ethanol, ethylene glycol, or detergents. An optimized *efe* gene from *Pseudomonas syringae*, encoding the ethylene-forming enzyme converting α-ketoglutarate into ethylene, has

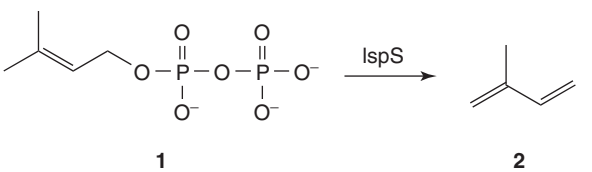

Figure 19.2 Synthesis of isoprene. Conversion of dimethylallyl pyrophosphate (1) into isoprene (2) by isoprene synthase.

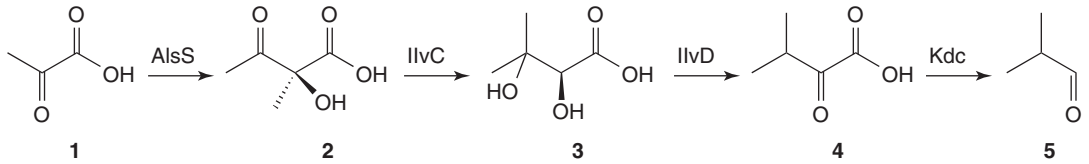

Figure 19.3 Synthesis of isobutyraldehyde. Conversion of pyruvate (1) into isobutyraldehyde (5) via 2-acetolactate (2) 2,3-dihydroxy-isovalerate (3) and 2-ketoisovalerate (4) For abbreviations of enzymes see text.

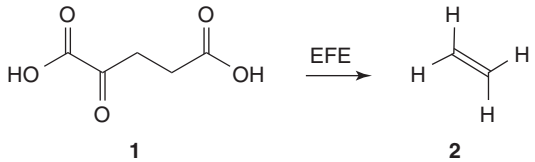

Figure 19.4 Synthesis of ethylene. Conversion of α-ketoglutarate (1) into ethylene (2) by ethylene-forming enzyme.

been expressed in *Synechocystis* sp. PCC 6803, yielding about 170 mg/l/day under optimized conditions (Figure 19.4; Ungerer *et al.*, 2012).

Sugars

Sugars are the natural products of photosynthesis. However, cyanobacteria, being photoautotrophic organisms and thus having limited need for sugar uptake into the cells, often lack the respective transporters, which are also needed for the excretion of hydrophilic metabolites. Under salt stress, *Synechococcus elongatus* PCC 7942 accumulates sucrose for osmoprotection. Introduction of invertase (*invA*) to convert the accumulated sucrose to glucose and fructose, in conjunction with introduction of a glucose- and fructose-facilitated diffusion transporter (*glf*; both genes from *Zymomonas mobilis*) led to the excretion of both glucose and fructose (Niederholtmeyer *et al.*, 2010).

Cellulose is used as feedstock for bioenergy production. Although to date the production rate achieved in *Synechococcus leopoliensis* engineered to express *Gluconacetobacter xylinus* cellulose synthase genes is too low for commercial exploitation, additional engineering efforts might improve the production rate (Nobles and Brown, 2008). For more information on cellulose production in cyanobacteria see Chapter 11.

Lactate

Lactate can be polymerized to yield polylactic acid, a biodegradable plastic with wide application, for example in medical implants and packing materials. Similar to the excretion of produced hydrophilic sugars, lactate also needs a transporter to be exported from the cells. Using *Synechococcus elongatus* PCC 7942 as the model organism, lactate dehydrogenase from *E. coli* was used to convert pyruvate to lactate, which was then exported by the cells using an *E. coli* co-transporter for lactate and protons (LldP; Niederholtmeyer *et al.*, 2010). Yields of lactate were reported to be about 50 mg/l/day/OD_{750}. The experiences made with this combination of producing/transporting proteins can possibly be extended to other hydrophilic metabolites such as succinate, fumarate, citrate, or amino acids (Ducat, Way, and Silver, 2011). *Bacillus subtilis* lactate dehydrogenase was introduced into *Synechocystis* sp. PCC 6803, and in conjunction with the heterologous expression of a soluble transhydrogenase a productivity of about 250 mg/l/day/OD_{750} unit has been reached (Figure 19.5; Angermayr, Paszota, and Hellingwerf, 2012).

Polyhydroxyalkanoate

Polyhydroxyalkanoates (PHA) are biodegradable polymers that can be used as a substitute for petrochemical-derived polyethylene in some applications, such as the manufacture of packaging films, containers, and paper

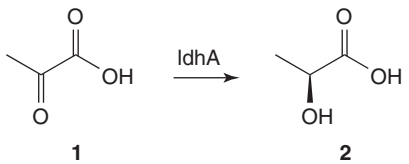

Figure 19.5 Synthesis of lactate. Conversion of pyruvate (1) into lactate (2) by lactate dehydrogenase.

coatings. They are naturally accumulated by various microorganisms as carbon storage. Cyanobacteria can be engineered to produce PHA from fixed carbon dioxide and accumulate it in amounts of more than 50% of the dry cell weight (Akiyama *et al.*, 2011).

19.4.3 High-value products

High value compounds from cyanobacteria comprise food additives such as polyunsaturated fatty acids (PUFA), vitamins, carotenoids and other antioxidants, pigments or colorants, and fine chemicals, making cyanobacterial products interesting for the food and cosmetic industries. The highest-value products from cyanobacteria are bioactive secondary metabolites, which serve as bioreagents or are used for agrochemical or pharmaceutical purposes. While the production of low-value products, especially biofuels, using recombinant cyanobacteria has been studied to some extent as described above, only few studies have been performed to date for high-value products.

19.4.3.1 Food additives

One of the first high-value products produced by engineered cyanobacteria was salmon growth hormone (Kawata *et al.*, 1991). Cyanobacteria producing this hormone can be fed directly to salmon, increasing their growth rates by up to 55% (Zang *et al.*, 2007).

Eicosapentaenoic acid (EPA) synthesis genes from EPA-producing *Shewanella* sp. SCRC-2738 have been successfully introduced into a marine *Synechococcus* strain. This resulted in the synthesis of the ω-3 PUFAs EPA and eicosatetraenoic acid (Yu *et al.*, 2000).

Astaxanthin is a commercially important carotenoid, responsible for the reddish color of salmonids and crustaceans. It is an extremely efficient antioxidant and may thus protect against reactive oxygen species, prevent cancer, and stimulate the immune system (Higuera-Ciapara, Félix-Valenzuela, and Goycoolea, 2006; Hussein *et al.*, 2006). The gene *crtO* from *Haematococcus pluvialis*, encoding β-C-4-oxygenase, which converts β-carotene to canthaxanthin, was introduced into *Synechococcus elongatus* PCC 7942 resulting in the production of astaxanthin (Harker and Hirschberg, 1997). Furthermore, it has been shown that it is possible to modify the carotenoid content and proportions of the individual carotenoids produced by cyanobacteria, and that plant-specific carotenoids can be produced into cyanobacterial hosts (Schäfer, 2003; Patsalo, Raleigh, and Green, 2011).

19.4.3.2 Pigments

Phycobiliproteins are part of the cyanobacterial light-harvesting system. As naturally fluorescent molecules, they are used as tags for cell sorting, in histological imaging, and in array technology applications (Kronick and Grossman, 1983; Sekar and Chandramohan, 2008). Recombinant C-phycocyanin subunits containing tags such as 6xHis, oligomerization domains, and the Strep2 tag have been produced in *Nostoc* sp. PCC 7120. These modified proteins can be used as fluorescent labels without the need of chemical manipulation of wild-type C-phycocyanin (Cai *et al.*, 2001).

19.4.3.3 Secondary metabolites

Cyanobacteria are well recognized producers of bioactive secondary metabolites, and in recent years, several excellent reviews have covered cyanobacterial metabolites (Gademann and Portmann, 2008; Tidgewell, Clark, and Gerwick, 2010; Niedermeyer and Brönstrup, 2012). For more details on the potential pharmaceutical uses of cyanobacterial secondary metabolites see Chapter 4.

Cyanobacterial natural products are chemically diverse (Guyot, Doré, and Devillers, 2004), but even though compounds from many chemical classes have been isolated, peptide and polyketide structural elements are predominant among cyanobacterial metabolites (Burja *et al.*, 2001; Welker and von Döhren, 2006; Tidgewell, Clark, and Gerwick, 2010). These compounds are often synthesized via combined polyketide synthases and non-ribosomal peptide synthetases (PKS/NRPS) (Dittman, Neilan, and Börner, 2001; Barrios-llerena, Burja, and Wright, 2007; Kalaitzis, Lauro, and Neilan, 2009). Although often not recognized at first glance, ribosomally synthesized products play an important role among bioactive metabolites from cyanobacteria as well (Ziemert *et al.*, 2008; Donia and Schmidt, 2010; Sivonen *et al.*, 2010).

As the biosynthetic pathways of peptide-derived natural products have been remarkably well studied, they are potentially amenable to directed mutation of

the encoding gene cluster, the aim being to increase the diversity of the respective biosynthetic pathway products. However, direct engineering of cyanobacterial secondary metabolism is largely in its infancy, and there is only one study describing the successful direct manipulation of the cyanobacterial biosynthetic machinery: the microcystin biosynthesis pathway of the freshwater cyanobacterium *Planktothrix* has been modified by deletion of the *O*-methyltransferase gene *mcyO*, resulting in microcystins lacking the O-methyl group of the Adda moiety (Christiansen *et al.*, 2003).

As many bioactive compounds are synthesized only in low amounts by cyanobacteria, research on the production of cyanobacterial secondary metabolites in heterologous hosts such as *E. coli* has been taken up. Proof-of-principle experiments have shown that it is possible to produce ribosomal metabolites in this way (Schmidt *et al.*, 2005; Donia, Ravel, and Schmidt, 2008; Ziemert *et al.*, 2010). Especially well studied is the heterologous expression of cyanovirin-N. Cyanovirin-N is a lectin comprising 101 amino acid residues, isolated from *Nostoc ellipsosporum* (Boyd *et al.*, 1997). It has been found to be a fusion inhibitor, preventing infection with all HI virus types. It is also strongly active against influenza A and B, respiratory syncytial virus and enteric viruses (O'Keefe *et al.*, 2003). As the compound is readily available using heterologous expression in *E. coli*, it has been discussed as a promising template for anti-HIV drugs (Jaspars and Lawton, 1998). Applications for cyanovirin-N are currently under investigation (Xiong, Fan, and Kitazato, 2010), although safety issues have been recognized (Huskens *et al.*, 2008). However, natural cyanovirin possesses only modest stability. Thus more stable variants retaining the affinity and specificity of natural cyanovirin have been rationally and computationally designed and expressed in *E. coli* (Patsalo, Raleigh, and Green, 2011).

Due to the size and multifunctional character of PKS/NRPS biosynthesis complexes, the heterologous expression of non-ribosomal cyanobacterial peptides is a significant challenge. Recent advances have shown that *Streptomyces* might serve as a suitable expression host for cyanobacterial PKS/NRPS genes (Jones *et al.*, 2012) and, very recently, there has been a report that heterologous expression of the barbamide biosynthetic gene cluster from *Moorea producens* in *Streptomyces venezuelae* results in the production of a novel barbamide congener (Kim *et al.*, 2012). Now that this milestone has been reached, it can be expected that research on heterologous expression of cyanobacterial metabolites will gain momentum.

In addition to heterologous expression of cyanobacterial peptides in non-cyanobacterial expression hosts, research on the more direct productivity optimization of the original producers, the cyanobacteria, remains an important task. In this regard, a patent has been filed describing *Synechocystis* sp. PCC 6803 as an expression host for secondary metabolites from the PKS/NRPS biosynthetic pathway (Neilan, Roberts, and Copp, 2010). However, successful examples of the use of this system have not been described in the scientific literature as yet, and in general there are no successful examples for the expression of cyanobacterial natural products in other cyanobacterial hosts.

Engineered *Synechococcus* and *Anabaena* strains transformed to produce mosquitocidal toxins from *Bacillus thuringiensis* have been discussed regarding their use against the cyanobacteria-ingesting *Anopheles* mosquito larvae – carriers of malaria – in their natural habitat (Sangthongpitag, Delaney, and Rogers, 1996; Ketseoglou and Bouwer, 2012). This is an example of cyanobacteria used as expression hosts for non-cyanobacterial natural products.

19.5 Concluding remarks and outlook

Although cyanobacteria are potentially cost-effective production organisms for a multitude of valuable compounds, there are only few studies substantiating the usefulness of genetic engineering for the production of high-value products in cyanobacteria. Especially for products such as proteins, PUFAs, vitamins, pigments, carbohydrates, and polysaccharides, previous studies have focused on variation and optimization of cultivation conditions rather than on genetic engineering (Sharma *et al.*, 2011).

As detailed above, this is different for low-value products, especially biofuels, where a multitude of patents and scientific studies have been published, highlighting successful cyanobacterial engineering. As the same set of molecular tools can be applied for

low- and high-value products, it can be expected that advances in engineering techniques on cyanobacteria will also lead to further studies related to high-value products.

In addition, recent extensive research and development of cyanobacteria-based biofuels and green chemicals has significantly helped in overcoming previous bottlenecks preventing the commercial exploitation of cyanobacteria, especially those related to their mass cultivation and product yields.

The progress achieved thus far makes cyanobacteria an attractive and still untapped source of low- and high-value products as well as an extremely useful host for the engineering of economically viable production systems.

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Chapter 20

Cryopreservation of cyanobacteria

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20.1 Introduction

Survival at low temperatures is a widespread phenomenon in nature and can be observed across a wide range of organisms including cyanobacteria (Fuller, Lane, and Benson, 2004; Fox, 2006; Thomas, 2005). Understanding survival mechanisms in nature (e.g., in permafrost) and in man-made "cryobanks" is important and will assist in the sustainable exploitation of biological resources for research and biotechnology.

Low temperature slows metabolism and under appropriate conditions enhances longevity, conferring stability on living cells. This attribute has been widely exploited by those who need to secure viable microbial biological resources in a stable condition (Kirsop and Doyle, 1991; Day and Stacey, 2007). However, the serial sub-culture of actively growing cells does not bestow the same level of stability and security as long-term cryogenic storage, or lyophilization (freeze-drying). Cultures may risk genetic selection, instability, loss of specific traits including the loss of capacity to produce phycobiliproteins, geosmin, and anatoxin *a* in cyanobacteria and contamination by other bacteria (Day *et al.*, 2007; Stacey and Day, 2007). Moreover, maintenance of active cultures is a costly, time-consuming, and resource-demanding practice. Successful storage of cyanobacterial strains in cryobanks is dependent upon the use of effective cryoprotective strategies and understanding the basic principles of cryobiology is fundamental in their development.

20.2 Cryopreservation: theory and practical application

The wide phenotypic and ecological diversity of cyanobacteria presents a challenge to the development of robust cryopreservation techniques. The application

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of effective storage protocols are underpinned by knowledge of:

- water behaviour
- cryoinjury
- cryoprotection.

Water exists in four states: as a liquid, glass, solid, and vapour, the formation of which is influenced by temperature. Manipulation of the liquid, glassy, and solid (ice) states of water is the main requirement to devise protocols that avoid the formation of lethal intracellular ice.

There are two main approaches to cryopreservation. The first is described as "traditional" or "conventional" and is often referred to as "controlled rate", "two-step", or "equilibrium" freezing. This involves the control of extracellular ice crystallization, a process described as nucleation, or "seeding" (the point at which ice crystals are initiated). It is a misconception that water freezes at 0°C, because when the environment prevents water molecules from aggregating, water can supercool to less than 0°C, with the lowest temperature possible for ultra-pure water being at, or around, −40°C (the point of homogeneous ice nucleation). During ice formation, energy is released as the latent heat of fusion. Conversely, ice melting is accompanied by an endothermic event.

The second approach to cryopreservation requires the cell to achieve a critically high viscosity, so that on exposure to freezing temperatures water forms a vitrified (glass-like) state. Glasses are in reality highly viscous solidified liquids. They are amorphous and, because of their lack of organized structure, are far less damaging to cells than crystaline ice. Glass formation involves thermal changes, of which the most significant is the glass-transition temperature (T_g), the temperature at which glass formation is initiated. Glasses are metastable and their behaviour in biological tissues is highly complex, because water can readily devitrify and convert back to ice. Knowledge of the thermal events associated with ice nucleation, melting, and glass transition are useful in developing cryopreservation protocols and specialist thermal analysis using a differential scanning calorimeter may be employed to study and optimize vitrification procedures (Harding *et al.*, 2004).

Tolerance to cryopreservation depends upon the ability to overcome or avoid cryoinjury induced by both physical and chemical changes associated with freezing. Mazur (1965) proposed that two factors are involved: ice formation and colligative damage. Ice promotes structural and osmotic damage, in particular causing mechanical injury to fragile cell structures (Fleck *et al.*, 2006). Colligative injury is induced by the excessive concentration of solutes, which is detrimental to cellular function. When slow-to-moderate cooling rates (generally <5°C/min) are applied, ice usually nucleates extracellularly, resulting in a vapor pressure deficit between the outside and inside of the cell; to maintain osmotic equilibrium water moves out of the cell. Rates of water loss are determined by the cooling rate, the dynamics of extracellular ice nucleation, the size and porosity of the cell, as well as cryoprotection. As cells shrink during the cryopreservation process their rate of water loss can be expressed as a function of cell size and cell permeability. The kinetics of water loss may be used to help predict their freezing (Mazur, 1965), and therefore mathematical equations and theoretical models of thermodynamic behaviour and membrane permeability (Kedem and Katchalsky, 1958; Rubinsky and Pegg, 1988; Chuenkhum and Cui, 2006) have been successfully employed to optimize controlled-rate cooling and cryoprotective protocols for animal and human cells (Gilmore *et al.*, 1995). However, their application in cyanobacteria is less relevant because of the presence of cell walls, extracellular mucilage and so on, which limit their applicability.

Successful conventional cryopreservation protocols are dependent upon optimizing cooling rates in conjunction with cryoprotective strategies. There are two main types of chemical cryoprotectants:

- penetrating/colligative
- non-penetrating/osmotic.

Traditional controlled-rate cooling protocols use penetrating cryoprotectants such as methanol, dimethyl sulfoxide (DMSO), or glycerol, generally in the concentration range 5–12% (v/v). As cells are exposed to controlled cooling (e.g. −1°C/min), ice forms extracellularly inducing the outward movement of intracellular water. This is desireable as it reduces the quantity of water molecules available that could potentially form lethal ice crystals. In many systems it is essential to induce extracellular ice nucleation (Fuller and Paynter, 2004), as from this

point onwards the operator has some control over the excursion of water. However, for cyanobacteria, controlled mechanical or thermal ice nucleation is rarely employed (Day and Brand, 2005). As water is removed, the freezing point becomes increasingly depressed allowing more time for water to exit the cell. Often a "hold" is programmed into controlled-cooling protocols (in the case of cyanobacteria generally 15 min), allowing additional water to move across the cell membrane. However, a balance must be achieved to ensure that dehydration does not result in an excessive, damaging concentration of solutes and this is where colligative cryoprotectants are effective. These chemicals penetrate cells and act as cellular solvents, thus protecting against the damaging concentration of solutes that occur as water is lost from the cell and additionally limit the deleterious and potentially lethal excessive reduction in cell volume. Following these treatments and when cells are exposed to terminal freezing temperatures and subsequently to liquid nitrogen, the intracellular solution is so concentrated/viscous that it vitrifies. Alternatively, if some water molecules remain available for nucleation, the ice crystals formed are so small they are inocuous.

To date there are no publications on the use of non-penetrating cryoprotectants to cryoconserve cyanobacteria. However, for completeness some information on their mode of action/efficacy has been included in this chapter. Non-penetrating cryoprotectants have multiple protective roles: they depress the freezing point, cause osmotic dehydration, and reduce the amount of water available for freezing. They may also impair ice nucleation by restricting the molecular mobility of water molecules (Fahy *et al.*, 2004; Fuller, 2004; Volk and Walters, 2006).

Cryopreservation using ultra-rapid rates of cooling necessitates a cryoprotective strategy that allows cells to be directly plunged into liquid nitrogen. This approach normally requires the formation and stabilization of a vitrified state, achieved by attaining a high cellular-contents viscosity, by evaporative and/or osmotic removal of water, the loading of high concentrations of penetrating cryoprotectants, or both. Vitrified systems are of two types;

- partially vitrified, where the solution external to the cells forms ice whilst the intracellular component remains ice free
- totally vitrified, where ice formation is inhibited in both the external and intracellular compartments.

Owing to their physical protective properties many commonly employed cryoprotectants also impart additional defenses against cryoinjury (Fuller, 2004; Benson and Bremner, 2004) as they can stabilize proteins and membranes and in some cases act as antioxidants.

Rewarming of cryopreserved samples normally involves relatively fast warming, commonly in the range 40–90°C/min. This prevents the "growth" of small ice crystals that are capable of growing to a size that may cause injury (Day, Watanabe, and Turner, 1998). Furthermore, relaxation of glasses on rewarming can fracture fragile cell structures or they can devitrify and form ice if they are rewarmed too slowly (Fleck, 1998).

20.3 Cryopreservation methodology

Cryoprotection is crucial to successful cryogenic storage, although some robust organisms, such as the unicellular eukaryotic alga *Chlorella protothecoides* (Morris, Clarke, and Clarke, 1977), survive without the need for cryoprotective additives or controlled cooling. However, the capacity to recover from cryopreservation without the need for chemical additives is not the case for most cyanobacterial strains, although some success has been observed in akinete-forming taxa (Lukesova and Day, unpublished observations). Cryopreservation of cyanobacteria is usually undertaken by controlled-rate cooling coupled with colligative cryoprotection, but vitrification has also been successfully employed. These approaches, as applied to cyanobacteria, are discussed in more detail below and some "standard" protocols are detailed in Table 20.1.

20.3.1 Controlled-rate cooling

Conventional cryopreservation protocols (Figure 20.1) based on regulating cooling rates, when employed for cyanobacterial conservation, usually apply a single colligative cryoprotectant such as DMSO, glycerol,

Table 20.1 Standard cryopreservation protocols used for cyanobacteria.

Origin of strains studied	Cryoprotectant	Cooling protocol		Reference
Freshwater	DMSO 5% (v/v)	(1 step) Direct storage at −80°C		Romo and Becares (1992)
Freshwater	DMSO 5% (w/v)	(2 step) −30°C for 15 min*	→ liquid N_2	Morris (1978)
Marine/hypersaline	DMSO 10% (w/v)	(2 step) −30°C for 15 min*	→ liquid N_2	Morris *et al.* (unpublished)
All	DMSO 10% (v/v)	(2 step) −1°C/min to −30°C	→ liquid N_2	Watanabe *et al.* (1992)
All	DMSO 10% (v/v)	(2 step) −1°C/min** to −40°C***	→ liquid N_2	Day and Brand (2005)
Terrestrial freshwater****	DMSO 8% (w/v)	(2 step) −1°C/min** to −40°C***	→ liquid N_2	Bodas *et al.* (1995)
Freshwater	Encapsulation in 1.2% alginate	Desiccation in 0.5 M sucrose	→ liquid N_2	Hirata *et al.* (1996)

DMSO, dimethyl sulfoxide; *immersion of cryovial in a pre-cooled alcohol bath (see text for detail); **achievable using either a passive freezer unit placed in a −80°C freezer, or a proprietary controlled-rate cooler (see text for detail); ***terminal temperature on employing a controlled rate cooler; ****cyanobacteria grown on agar slope in cryotube, overlayered with cryoprotecrant.

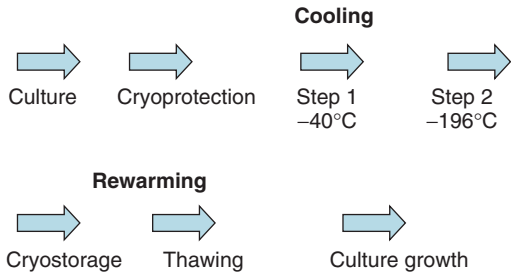

Figure 20.1 Generic two-step cryopreservation protocol.

or methanol (Holm-Hansen, 1973; Box, 1988; Bodas *et al.*, 1995; Day and Brand, 2005) followed by cooling steps alternated with the manual, automatic, or passive induction of ice nucleation (Day and Brand, 2005). Survival can be improved by pre-culture and cold hardening treatments applied prior to cryopreservation, which usually simulate natural acclimation responses. Examples include culturing algal cells at lower-than-normal growth temperatures (Morris, 1976), providing biochemical additives to enhance stress tolerance in plants (Luo and Reed, 1997), or the incorporation of novel antioxidants such as desferrioxamine (Fleck *et al.*, 2000). Furthermore, the collapsing of gas vacuoles in planktonic cyanobacteria has been demonstrated to enhance post-preservation survival (unpublished observations). In the case of cyanobacteria there have been very few systematic studies exploring these factors that are critical to controlled-cooling methods. However, it is well known that different cell types have specific optimal cooling rates, which are largely empirically determined (Mazur, 1965; Day and Brand, 2005). Complex cooling regimes, capable of more effectively dissipating the latent heat of fusion on ice formation may be beneficial particularly for strains with larger cells, as well as more complex or colonial taxa (Day and Brand, 2005). Computerized, controlled-rate programmable freezers provide the most reliable means of controlling cooling parameters, which in sequence are:

- Loading temperature (ambient/sub-zero centigrade; most commonly ambient for cyanobacteria).
- The first cooling ramp (−Y°/min; most commonly 1°/min for cyanobacteria).
- Temperature of initiating ice nucleation (−Z°C) with an optional hold (often omitted for cyanobacteria).
- A second or third optional cooling ramp (−Y°/min; often omitted for cyanobacteria).
- An intermediate terminal transfer temperature at, or near the point of homogeneous ice nucleation (approx. −40°C).
- A hold (X min; often 15 min for cyanobacteria).
- Transfer to liquid nitrogen.
- Storage in liquid- or vapor-phase liquid nitrogen.
- Optimized rewarming using rapid (40–75°C) or two-step approach involving holding at room temperature for 1–2 min, followed by rapid warming (most commonly using a water-bath pre-warmed to 40°C for cyanobacteria).

In most cases effective protocols have been developed empirically, by varying the above parameters (see references cited in Table 20.1). Computerized programmable freezers have assisted in this process, because they provide data outputs allowing each run to be documented for protocol development and quality-control purposes. Programmable freezers accurately regulate cooling rates over a broad range of temperatures by feeding pressurized vapour-phase liquid nitrogen into a sample chamber via an electronically-controlled, solenoid valve feedback system. Where it is necessary to manipulate cryodehydration, for example following extracellular ice nucleation, some machines have, or can be fitted with, an automatic seeding facility. Furthermore, "dwell periods" may be built into the program to facilitate additional freeze-dehydration and to maintain the chamber temperature whilst performing transfers of the vials to liquid nitrogen. It is important to transfer the cryogenic vial quickly from the cooling chamber to the storage vessel to avoid any significant sample re-warming that could induce devitrification or freeze-fracture events.

Alternatives to liquid-nitrogen-cooled, electronically programmed freezers may also be employed to achieve the desired cooling protocol. These include passive coolers that rely on the thermal conductivity of an insulated containment vessel placed in an electrical deep-freeze, refrigerator, and/or a solvent bath. Simple cooling ramps can be developed by placing cryovials in a polystyrene box ($\sim 10 \times 10 \times 10$ cm internal dimensions with walls and lid ~ 3.5 cm thick) placed in a -80°C freezer. The insulated container retards heat transfer, allowing the contents of the cryogenic vial to cool relatively slowly (-1°C/min). Undisturbed, the sample reaches the ambient temperature, at a specific rate of cooling that is dependent on the insulation properties of the container. These systems can be calibrated over a time-course using thermometers, thermocouples, and simple data-logging devices. Over a narrow range of temperatures passive cooling systems have a near-linear cooling rate (Benson, 2008). Additionally, the properties of a cooling vessel or refrigerated chamber can be modulated by adding a solvent such as isopropyl alcohol. This is the basis for commercial systems that use solvent-containing units to passively cool at approximately -1°C/min from 0°C to -40°C (e.g., Mr. Frosty, Nalgene Nunc, Rochester, NY, USA). Alternatively, samples can be cooled using direct immersion in a denatured-ethanol refrigerated bath (Day and Brand, 2005). When the sample reaches a sufficiently low temperature (usually between -30 and -80°C), it is removed and plunged into liquid nitrogen. The conventional approach to controlled-rate cooling cryopreservation creates an approximately linear change of temperature with time during the first cooling ramp. Morris and co-workers (1999) demonstrated that human spermatozoa can be successfully cryopreserved by manipulating the cooling regime to provide non-linear changes in temperature with time over critical temperatures. This approach has been successfully employed for euglenoid algae, but has not been tested for cyanobacteria (Morris and Day, unpublished observation). Modifications to post-storage culture can also significantly enhance the level and rate of recovery and in general it is optimal to allow a period (~ 24 h) in the dark post-warming (Day and Brand, 2005).

In most cases the above approaches have been employed to conserve liquid cultures of cyanobacteria. However, the same technique has been used to cryopreserve cyanobacteria cultured on agar slopes, overlayered with a cryoprotective solution of 8% DMSO, in 2 ml cryovials (Bodas *et al.*, 1995). This approach has also been employed to preserve cyanobacterial cultures on agar plates (Watanabe, Tsuzuki, and Sugiyama, 1994).

20.3.2 Vitrification

At the glass-transition temperature (T_g), molecular motion ceases and a liquid becomes a glassy solid. In controlled-rate cooling it is possible that on exposure to freezing temperatures the intracellular viscosity of the cell is sufficiently high that cells become vitrified, whilst remaining in a frozen extracellular environment. This is a partially vitrified system, although ice nucleation is still required to evoke osmotic, freeze-induced dehydration. Ice-free cryopreservation, initially pioneered for animal cells (Fahy *et al.*, 1984) has been applied to eukaryotic micro-algae and cyanobacteria (Harding *et al.*, 2004). This involves a different cryoprotective strategy, in which ice formation is inhibited both inside and outside the cell, resulting in total vitrification and thus obviating any potential damage induced by crystaline ice formation. Vitrification does not require

controlled cooling, and the need for programmable freezing apparatus is circumvented, making cryopreservation more amenable to researchers from non-specialist laboratories. However, as glass formation usually requires excessive concentration of solutes, cells must be able to tolerate dehydration. Approaches to achieve vitrified and frozen states are frequently interdependent and "hybrid" protocols are increasingly being developed; these incorporate components of colligative cryoprotection/cooling and vitrification procedures, although their final end point is usually the glassy state (Benson, 2008).

The "conventional" approach to vitrification developed for human and animal systems utilizes mixtures of "traditional" cryoprotectants (DMSO, glycerol, ethylene glycol, polyols, and sugars) at high (>25% v/v) concentrations. However, commonly used vitrification solutions, of which Plant Vitrification Solution Number 2 is the most widely used (Sakai, 2004), are invariably toxic to microalgae and cyanobacteria (Fleck, 1998). Alternative approaches to remove intracellular water, such as encapsulation/dehydration (Fabre and Dereuddre, 1990), may achieve the same effect. This method, in which cells are entrapped in calcium-alginate beads, involves osmotic dehydration in high molarity sucrose solutions, then evaporative desiccation in a sterile air-flow or over silica-gel. The sugar-loaded alginate matrix supports very stable glasses so long as the system is desiccated to below a critical moisture content (~10–15% w/v) that inhibits devitrification on rewarming as well as cooling (Benson *et al.*, 1996; Harding *et al.*, 2004). This approach has been sucessfully used by Hirata *et al.* (1996) to conserve filamentous freshwater cyanobacteria (Table 20.1), but has not been widely adopted because conventional methodologies are simpler to employ and equally effective for non-recalcitrant taxa.

20.3.3 Storage

In many organizations such as the Culture Collection of Algae and Protozoa, cryo-storage is in liquid-phase liquid nitrogen at −196°C, but vapour-phase storage, where temperatures may vary between −196°C and −135°C in the cryostat, is perfectly satisfactory for materials cryopreserved by conventional methodologies. Under this storage regime viability levels should remain unchanged for decades, if not longer (Grout, 1995), and the author has not observed any reduction in viability in cyanobacterial samples stored for over 15 years (Day and Watanabe, unpublished observations). However, for samples cryopreserved employing vitrification approaches, devitrification and subsequent formation of crystalline ice may occur at relatively low sub-zero temperatures (Benson, 2008), so liquid-phase storage is better since this will ensure thermal stability of the samples.

Unlike eukaryotic microalgae (Holm-Hansen, 1973; Day and Brand, 2005), cyanobacteria can be successfully stored for long periods at −80°C, thus increasing the applicability of the approach to labs where ultra-low-temperature storage facilities are not available.

20.4 Cryopreserving biological resources

Cryogenic storage ensures the safe, long-term security of micro-organisms, including cyanobacteria, of both present and potential economic value. There is a major financial incentive to cryopreserve high-value microorganisms, which had estimated global annual sales of US $30–60 billion in 1999 (Kate and Laird, 1999). This is particularly the case for those strains used or likely to be used by the pharmaceutical sector for the production of drugs and other healthcare products. Cyanobacteria have been successfully explored for these types of products, with multiple leads on novel antimicrobials and anticancer agents in particular (Pulz and Gross, 2004; Singh, Kate, and Banerjee, 2005). Cryogenic storage of microorganisms exploited by the biotechnology industries underpins the absolute requirement of this sector for phenotypic and genotypic stability in master stock-cultures used for patenting, commercialization and sustainable exploitation. Moreover, modern biotechnological approaches to drug discovery, such as bioprospecting, genetic manipulation, genomics, and proteomics have increased the need to develop robust cryopreservation protocols that safeguard genetic materials. This will become increasingly important as the cyanobacterial biotechnology sector develops and matures, as there will be greater effort expended on developing "elite" strains, either conventionally or through genetic manipulation. Whilst

for some cyanobacteria, lyophilization is an alternative option to cryogenic storage (Day *et al.*, 2007; Silva, Ferrari, and Silva, 2007), preservation in liquid nitrogen should be the preferred approach. This is because the stability of production of primary and secondary metabolites has been confirmed after ultra-low-temperature storage (Hédoin *et al.*, 2006; Wood *et al.*, 2008), whereas, although there are no data for cyanobacteria, production losses of secondary metabolites have been reported in suboptimally lyophilized fungi (Ryan *et al.*, 2003).

20.5 Management of cryopreserved resources: biological resource centres

Cyanobacterial cultures are held in formal or informal collections located in academic, public-service, private, government, and commercial organizations worldwide. With some exceptions, cryopreserved "collections" are housed in one of the major service-based biological resource centres (BRCs), including:

- The American Type Culture Collection http://www.lgcstandards-atcc.org/
- The Culture Collection of Algae and Protozoa http://www.ccap.ac.uk
- The National Institute for Environmental Science collection http://mcc.nies.go.jp/;
- The Pasteur Culture Collection of Cyanobacteria http://www.pasteur.fr/ip/easysite/pasteur/en/research/collections/crbip/general-informations-concerning-the-collections/iv-the-open-collections/iv-iii-pasteur-culture-collection-of-cyanobacteria;
- The Sammlung von Algenkulturen Göttingen http://sagdb.uni-goettingen.de/
- The University of Texas collection http://web.biosci.utexas.edu/utex/.

These collections perform a key role in delivering documented, characterized cultures as "seed stocks" for:

- use in medicine, agriculture, or bioindustry
- as reference strains for biological assays and published scientific literature
- as type strains for taxonomical studies
- as centres for conservation of biodiversity.

BRCs maintain practices, including the application of optimal cryopreservation protocols, to ensure that the samples of organisms they hold and distribute sustain these important attributes. In addition, for those organizations dealing with microorganisms and biomedical materials, master and distribution banks for each organism and robust quality-control systems are normally established (Stacey and Day, 2007). It is particularly important that high standards are maintained in facilities handling genetically manipulated organisms and in patent depositories, where the preserved cells must remain viable for at least 20 years (Budapest Treaty, 1977). Security and stability of stored material is assured through adoption of appropriate management systems to restrict access to authorized personnel, appropriate alarms for nitrogen storage vessels, and documented procedures for filling and maintenance of nitrogen storage. Monitoring using temperature-alarm systems and auditing to ensure correct maintenance and documentation are also important activities for BRC operations.

Accurate records are vital to enable retrieval of the stored ampoules. Proprietary database systems have been specially designed for this purpose, but it is important to select a system that is flexible to the full range of user requirements. It is also sensible to have an up-to-date hard-copy version or back-up electronic copies, and if possible to ensure that amendments to storage records can be made at the storage site to avoid transcriptional errors.

In conclusion, there are clear commercial and scientific drivers for the implementation of storage of biological resources for reference or future exploitation. Cryopreservation provides the most appropriate and widely applicable approach to the long-term conservation of cyanobacterial strains. There remain some constraints on the materials that can be successfully held. However, new cryoprotective approaches have considerably extended the range and diversity of taxa, and in some cases the levels of post-thaw viability, of cryopreserved cyanobacteria. Ongoing challenges include the preservation of more structurally complex taxa – those with large amounts of mucilage, many-gas-vacuolated strains, and non-axenic isolates –where the levels of commensal bacteria and/or fungi are relatively high.

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Chapter 21

Patents on cyanobacteria and cyanobacterial products and uses

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21.1 Introduction

Patents represent a detailed source of information on potentially commercial products and processes and some of the latest science and technology. Because an essential requirement of being granted a patent is that the information disclosed therein has not been made public before the patent is applied for, patents are an important source of information on the latest developments. Therefore, the patent literature is as important as the scientific literature as a source of information on research and development. Since patents also need to cite relevant literature, they are also a good source of relevant papers published in the field.

Patents are public documents and effectively are part of the "open access" literature. They also represent the most current literature on developments with commercial potential. For an invention to be patentable the work must not have been publicly available before the patent application is lodged: none of the information can have been published or presented at conferences or other seminars and so on. The patent literature also provides a unique overview of the developments and trends in the commercial application of cyanobacteria.

Searches of the patent literature are now very easy because patents can be found on-line. Key searchable patents databases are the US Patents and Trademarks Office (USPTO; www.uspto.gov), the European Patents Office (EPO; www.epo.org), and other country-based patents offices such as those in Australia and Canada. The Espacenet searchable database of the EPO (www.espacenet.com/) is an excellent place to search patent applications worldwide and even Google provides a search of USPTO patents and patent applications.

It is important to note the difference between patent applications and granted patents. Many applications never proceed to the granting stage, sometimes because

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the applicants choose not to proceed to the examination stage. The granting of a patent can take many years. Knowing whether a patent has been granted is complicated because the USPTO uses a different numbering system for patent applications and patents, whereas the EPO maintains the same number, changing only the letters after the number (A1, A2 and so on for applications and B1, B2 for granted patents). The World Intellectual Property Organisation provides a unified procedure for filing patent applications under the Patent Cooperation Treaty (PCT), which is an international patent law treaty. A PCT application does not itself result in the grant of a patent, since there is no such thing as an "international patent,", the grant of patent being a prerogative of each national or regional authority.

Two recent papers have reported in some detail on the results of searches and analysis of the patent literature on cyanobacteria. Sekar and Pulraj (2007) searched the USPTO patent database from 1976 to April 2006 and found 265 patents of relevance. Sekar and Chandramohan (2008) also reviewed the literature and patents on the production and applications of phycobiliproteins. This chapter will focus on patenting activity with respect to cyanobacteria since about 2008. This chapter mainly concerns US patents and PCT patent applications, although some other country-specific patents and applications are included.

The patents and patent applications cited here are arranged in broad groupings for clarity, but there is some overlap at times, especially when the patents make broad claims.

21.2 Cyanobacteria and the environment

Two recent patent applications describe methods for controlling cyanobacterial blooms. Zbořil, Mašláň, and Maršálek (2012) describe the use of ultra-small iron particles to remove, prevent, and reduce the expansion of cyanobacterial blooms, while Bogdavov (2012) claims that the planktonic green alga *Parachlorella nurekis* can be used to eliminate cyanobacteria, other bacteria, and fungi. Several other patents are concerned with the detection of toxic cyanobacteria, particularly toxic *Microcystis* spp. (Neilan and Jungbult, 2006; Sivonen *et al.*, 2007; Oh *et al.*, 2008a; Neilan *et al.*, 2011). The methods described include either the use of genetic markers and probes, or immunology-based methods (Lopez Rodas *et al.*, 2010). A method for detecting cyanobacterial toxins has also been patented (Oehrle, 2011).

Several applications and methods of using cyanobacteria to control land erosion or as fertilizers are described in the patent applications of Yang and Choi (2007), Flynn (2008), and Marler (2009).

An interesting recent patent application is that of Blanchet *et al.* (2012) who describe a process of sequestering atmospheric carbon dioxide by culturing unspecified cyanobacteria so that precipitation of calcite occurs. The calcite "fossilizes" the carbon dioxide and the organic biomass can be further processed, for example by fermentation, to produce methane as an energy source. This concept for carbon dioxide bioremediation is similar to that of Moheimani and Borowitzka (2006), who used the coccolithophorid alga *Pleurochrysis*. The culture of cyanobacteria for carbon dioxide sequestration is also described by Garcia Guerrero *et al.* (2006).

There has been increased application of genetic engineering to produce improved cyanobacterial strains for various industrial applications (see below), but there are potential risks associated with escape of these genetically-modified cyanobacteria into the environment. Possible solutions for this problem are addressed in several patents (Eisenstadt *et al.*, 2010; Chen *et al.*, 2011, 2012).

The patent application by Kramer *et al.* provides a method and kit using molecular probes for the identification of cyanobacteria (Kramer, Meixner, and Lindner, 2010).

21.3 Cyanobacteria products and applications

The use of cyanobacteria as sources of bioactive molecules and other products has been a subject of very active patenting activity in the past (Sekar and Pauraj, 2007), but in the last few years patenting activity in this area has decreased. However, patents for a wide range of both pharmaceutical (Tadros, 2009; Jensen

and Drapeu, 2010; Molteni, 2011; Scoglio *et al.*, 2011; Chiuh *et al.*, 2012) and cosmetic applications (Hours and Bruneau, 2009; Brune and Oreal, 2010; Einarsson, Brynjolfsdottir, and Krutman, 2010; Zirwen and Kleen, 2010; Capello, 2012; Molteni, 2012) have been applied for. Some specific products include a mycosporine-like amino acid, incorporating a glycosyl group, for possible use as a sunscreen (Sakakibara, Torii, and Miyamoto, 2009), and the overproduction of the antioxidants ubiquinone and ubiquinol (Dürr, 2012).

Several patents concern the production, extraction, and purification of carotenoids (Weiss A. *et al.*, 2007) or phycobilin pigments from cyanobacteria (Berjermo Roma and Acien Fernandez, 2009; Heyde, Schiffelbein, and Christiansen, 2012) as well as the use of these phycobilin pigments such as allophycocyanin in creating fluorescent conjugates for use as fluorescent markers (Geiger *et al.*, 2010). Other product patents include that of Mohammed and Vermaas (2009) who describe genetically engineered *Synechococcus*, *Synechocystis*, *Spirulina (Arthrospira) platensis*, *Trichodesmium*, and *Crocosphaera* overproducers of polyhydroxyalkonate and cyanophycin for the production of bioplastics and biomaterials, and that of Nobles and Brown (2008b), who describe cellulose production from the halophilic cyanobacteria *Agmenellum quadriplicatum* UTEX B2268 and *Synecochoccus* spp. PCC7002 and ATCC27264. Fatty alcohols are also of interest, because they and their derivatives have numerous commercial applications, including uses as surfactants, lubricants, plasticizers, solvents, emulsifiers, emollients, thickeners, flavors, fragrances, and fuels. Roessler, Watts, and Liu (2011) and Lu *et al.* (2011) have applied for patents to produce these using GM cyanobacteria and other microorganisms.

Zhou and Gibbons (2012) describe several *Anabaena* spp. that have been genetically engineered to have increased production of certain carbon-based products such as ethanol, linolool, myrcene, or farnesine. These genetically engineered hosts efficiently convert carbon dioxide and light into carbon-based products. Several constructs containing polynucleotide-encoding enzymes active in the metabolic pathways of cyanobacteria are disclosed. Furthermore, several of the strains have been further genetically modified to optimize production of the products. The optimization includes both upregulation and downregulation of particular genes.

New bioactive compounds from cyanobacteria recently patented are:

- coibamide A, with anticancer activity, from a *Leptolyngbya* sp. (McPhail *et al.*, 2011)
- MVL, an antiviral protein, from *Microcystis viridis* (Bewley, 2011)
- the cyanobacterial peptides, the scytovirins, originally isolated from *Scytonema varium* (Boyd *et al.*, 2009), and variants of the cyanovirins originally isolated from *Nostoc ellipsosporum* (Snell *et al.*, 2009; Boyd, 2010), both with application as antiviral compounds
- *Anabaena variabilis* phenylalanine ammonia-lyase (AvPAL) variants and compounds derived from these for use in compositions for cancer treatment (Kakkis *et al.*, 2009, 2010).

The gene cluster for the microginins, which are ACE inhibitors, has been cloned from *Microcystis aeruguinosa*, with possible application in the treatment of hypertension and patented for the biosynthesis of these compounds (Kramer, 2010).

The extraction and purification of the phycotoxins neosaxitoxin, saxitoxin, and golyaulatoxins 2 and 3 from *Cylindrospermopsis*, *Microcystis*, *Anabaena*, *Gomphosphaeria*, *Oscillatoria*, *Aphanizomenon*, and *Lyngbya wollei* is described by Lagos Gonzalez (2010), and Laguette (2011) describes a method of rapid extraction of hepatotoxins from cyanobacteria. Ehmann and Guthrie (2012) describe a method for removing contaminating microcystin toxins during the purification of phycocyanin from cyanobacterial extracts.

Genetic engineering methods for algae (including the cyanobacteria) are being developed for the production of therapeutic proteins (Weiss E. and Desplancq, 2009; Rasala *et al.*, 2011) and the patent by Bekker, Teixeira de Mattos, and Hellingwerf (2011) describes L-lactate production by a recombinant *Synechocystis* sp. Ufaz and Gressel (2010) claim a method for producing genetically modified cyanobacteria (incl. *Synechococcus*) and algae with an improved lysine and methionine content for use as animal feed.

A rather novel application of cyanobacteria is in their use in a bio-based electricity generating system (Bombelli, 2010). Equally novel is the use of cyanobacteria or parts thereof in optoelectronic devices, where

the cyanobacteria or the photosynthetic reaction-center protein are bonded to metal surfaces and then generate a current when illuminated (Frolov *et al.*, 2005; Carmeli *et al.*, 2011, 2012).

21.4 Cyanobacteria and biofuels

By far the largest number of recent patents relate to improving cyanobacteria by genetic engineering for the production of biofuels.

Hydrogen production from cyanobacteria has received relatively little interest recently. The only recent patent applications are those by Nelson *et al.* (2010), Xu and Smith (2010), Lee (2010c), and Silver, Savage, and Agapakis (2012). The patent of Spormann and Ng (2010) concerns the expression of functional, heterologous oxygen-sensitive proteins – for example a hydrogenase from *Clostridium* – in filamentous cyanobacteria such as *Anabaena variabilis* and *Nostoc punctiforme*. An alternative and original approach to generate energy from photosynthesis is that of Kistenmacher *et al.* (2011), who engineered cyanobacteria to overproduce ammonium as a "hydrogen carrier."

A method of ethanol production by growing cyanobacteria and then fermenting the sugars to produce ethanol has been claimed by Pyo (2010). Several patents concern the development of methods of genetically engineering cyanobacteria to produce ethanol, the earliest of which seems to be that of Woods, Coleman, and Deng (2004) who introduced and expressed DNA-fragments from the yeast *Zygomonas mobilis* encoding for pyruvate decarboxylase and alcohol dehydrogenase. Recent patent applications or patents on GM ethanol-producing cyanobacteria are those of Fu (Fu and Dexter, 2007; Fu, 2010, 2012), Dehring, Kramer, and Ziegler (2012) and Green, Reppas, and Robertson (2011a, b); for a review see also the recent book chapter by Lee (2013). The production and excretion of ethanol in particular, has the attraction that the ethanol can be collected without the need to harvest and extract the cyanobacterial cells (Lee, 2010b), and a photobioreactor system to do this has been invented (Woods *et al.*, 2010). Methods to create genetically modified cyanobacteria producing butanol have also been invented (Lee, 2010a, 2012), as have other processes to produce butanol using cyanobacteria (Grady, Jahic, and Patnaik, 2011).

The production of other volatile compounds is also being developed. For example, Melis and Lindberg (2010) and Melis (2012) describe a method for an expression cassette and DNA sequence encoding for isoprene synthase and introducing this into *Synechococcus* sp. so that the cyanobacterium produces the volatile compounds isoprene and methyl-butenol, which could potentially be recovered in a manner similar to the ethanol described above. The patent application by Roessler, Liu, and Kristof (2012) claims a recombinant photosynthetic microorganism comprising at least one heterologous nucleic acid sequence that encodes a branched-chain 2-ketoacid decarboxylase so that the organism can produce branched-chain alcohols including 2-methyl-1-butanol, 3-methyl-1-butanol, and isobutanol.

There has also been strong patenting activity in the area of increasing the production of lipids and isoprene hydrocarbons, where methods to genetically modify cyanobacteria and other algae have been developed. The patent application by Anderson (2008) claims the production of isoprene using a metabolically engineered *Anabaena*. Champagne and Kuehnle (2009) claim methods and gene constructs that encode a gene of interest that encodes a molecule selected from the group consisting of IPP isomerase, acetyl-coA synthetase, pyruvate dehydrogenase, pyruvate decarboxylase, acetyl-coA carboxylase, α-carboxyltransferase, β-carboxyltransferase, biotin carboxylase, biotin carboxyl carrier protein and acyl-ACP thioesterase, beta ketoacyl-ACP synthase, FatB, and a protein that participates in fatty acid biosynthesis via the pyruvate dehydrogenase complex. These gene constructs may have application in the production of biofuels and other products from cyanobacteria and other algae. Liui and Curtiss (2010) modified cyanobacteria to increase their content of neutral lipids, while Kay *et al.* (2012) claim fatty-acid-producing cyanobacteria that secrete these compounds into the medium.

Roberts *et al.* (2011) claim modified cyanobacteria that contain one or more exogenous genes encoding diacylglycerol acyltransferase, a phosphatodate phosphotase, and/or an acetyl-CoA carboxylase capable of

producing triglycerides as well as organisms with mutations or deletions in a glycogen biosynthesis or storage pathway that causes them to accumulate reduced amounts of glycogen under nitrogen-deficient conditions (Roberts *et al.*, 2010a, b). Reppas and Ridley (2010, 2011; Ridley and Reppas, 2011, 2012) claim methods of genetically modifying cyanobacteria (e.g., *Synechococcus*) including using recombinant l-alkene synthetases, acyl-ACP reductase, alkenal decarboxylase monooxygenase and so on, and other methods to produce alkenes and alkanes, such as l-nonadecene, l-octadecene, and n-pentadecane. Genetically modified cyanobacteria and algae expressing proteins that include transcription factor domains for upregulation of lipid biosynthesis pathways have been patented by Kristof and Sheshadri (2012).

There is also increasing activity in the development of algae and cyanobacteria that excrete particular products (e.g., Nobles and Brown, 2008a; Roessler *et al.*, 2009; Way *et al.*, 2011; Kay *et al.*, 2012; Reppas, 2012).

The patent application by Vermaas (2012) is wide reaching and concerns providing photoautotrophic bacteria, including cyanobacteria, that have been modified to introduce, delete, and/or alter the sequence or expression level of gene(s) of interest to increase the production of a range of desired products. The desired product can be processed into useful products such as biofuels, bioplastics, animal feed additives, valuable pigments, anti-oxidants, or organic fertilizers.

There are also a few patents employing non-genetically modified cyanobacteria for biofuels production.

21.5 Culture systems, processes, harvesting and processing

There are many examples in the patent literature of methods and systems for growing algae and cyanobacteria and their recovery and processing. Here only a few examples have been selected. For example, Oyler (2010a) has invented a closed-loop system in which the cyanobacteria are cultured in a reactor, harvested, and the biomass is gasified to produce methane and carbon dioxide, which is then recycled into the growing system after gasification. Mancinelli and Smernoff (2012) describe a system where cyanobacteria and yeast are co-cultured to produce an animal feedstock. A similar concept is illustrated in the patent application of Koshland (2011).

Systems continue to be developed to increase the light available for the cyanobacteria and algae in the culture system (Willson *et al.*, 2008). Amongst the more interesting of these are the use of LEDs (Dodd *et al.*, 2012), the use of fluorescent material to convert more of the available light to wavelengths usable by algal photosynthesis and arranged in such a way as to act as a light guide (Falber, 2012), and the use of genetically engineered cyanobacteria expressing a fluorescent protein so as to absorb UV and near-UV wavelengths and emit the light at useable wavelengths (Einbinder *et al.*, 2010).

Genetically engineered cyanobacteria with either herbicide resistance to assist in the maintenance of pure cultures (Chen *et al.*, 2010) or cyanobacteria containing introgressed virus/phage DNA to protect against viruses/phages (Chen, Danon, and Gressel, 2010) have been the subject of patent applications. The application by Yuan (2012) provides new biocides, methoprene and azadirachtin, for controlling grazers.

Cyanobacteria have also been modified to make their culture and harvesting easier. For example, the patent by Devroe *et al.* (2010) describes an engineered *Synechococcus* sp. PCC 7002 which no longer requires vitamin B12 for growth, making it easier and cheaper to culture; the patent application by Oh *et al.* (2008b) claims a strain of *S. platensis* that clumps and floats.

Different nutrient sources and substrata for growing cyanobacteria are also being patented. The use of chitosan as a nitrogen source for cyanobacterial cultures is the subject of a patent application by Blank and Hinman (2012), while Evans (2012) has applied for a patent for methods for growing cyanobacteria on bacterial cellulose. The co-culture of nitrogen-fixing cyanobacteria with other algae has also been described (Dhamwichukorn, 2011).

There are two recent patents on harvesting algae and cyanobacteria (Schlesinger *et al.*, 2011; Youngs, Cook, and Rogers, 2011). Hebner *et al.* (2012) describe

an electromechanical method of lysing algal and cyanobacterial cells, and Oyler (2010b) also describes a process of oil recovery by rupturing the algal cells.

21.6 Cyanobacterial genes in action

Specific cyanobacterial genes for various applications have also been the subject of patenting activity. For example, Papes and Arruda (2012) have characterized an active *Anabaena* sp. sucrose synthase gene, which it is claimed can be used to engineer trees and other plants to produce more cellulose. Similarly, Kakefuda and co-inventors (Kakefuda *et al.*, 2009) describe cyanobacteria genes from *Synechocystis* and *Anabaena* that can be used to modify plant traits including herbicide resistance; Takabe, Takabe, and Uchida (2010) describe a DNA sequence derived from the halophilic cyanobacterium *Aphanothece halophytica* that encodes for a heat shock protein, such as a DnaK protein or an HSP70 protein, and its use to impart drought tolerance to plants. Another application of cyanobacterial genes is described in the application by Kehoe, Li, and Alvey (2010), who claim light-regulated promoters from *Synechocystis* and *Fremyella diplosiphon* that can be used to regulate transcription in *E. coli* and other prokaryotes.

21.7 Concluding remarks

The potential of cyanobacteria in the production of useful chemicals and bioactive compounds continues to be strongly reflected in the ongoing patenting activity described above. Furthermore cyanobacteria also have applications in land management as soil conditioners and possibly also in mitigating atmospheric carbon dioxide and as supplements in animal feed. Together with other algal compounds and extracts, cyanobacteria also feature in cosmetics and nutraceutical applications. Finally, the detection and management of toxic cyanobacterial blooms remains a challenging issue. Overall, patenting activity with respect to cyanobacteria continues to increase each year and the patent literature remains a very informative source on the applied research and development of these organisms.

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Plate 5.3 View of the exterior of stone/mortar monuments at Santiniketan, West Bengal, India showing colonization of phototropic biofilms imparting blackish coloration to the substratum.

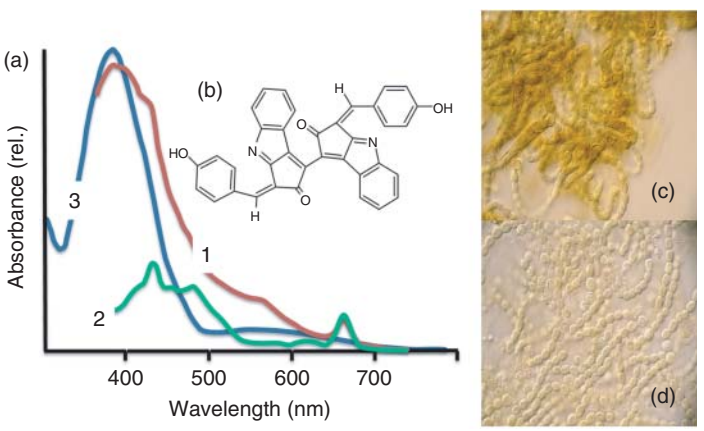

Plate 8.3 (a) Absorption spectrum of lipid-soluble pigments from the wild type *Nostoc punctiforme* ATCC 29133 (spectrum 1) and the scytonemin-deficient mutant (spectrum 2) exposed to UVA; note the absorption of scytonemin at 384 nm in the wild type. (b) The structure of scytonemin and its spectrum in solution (spectrum 3). (c) Images of the wild type and (d) scytonemin-deficient mutant exposed to UVA; note the accumulation of scytonemin as a brown pigment in c.

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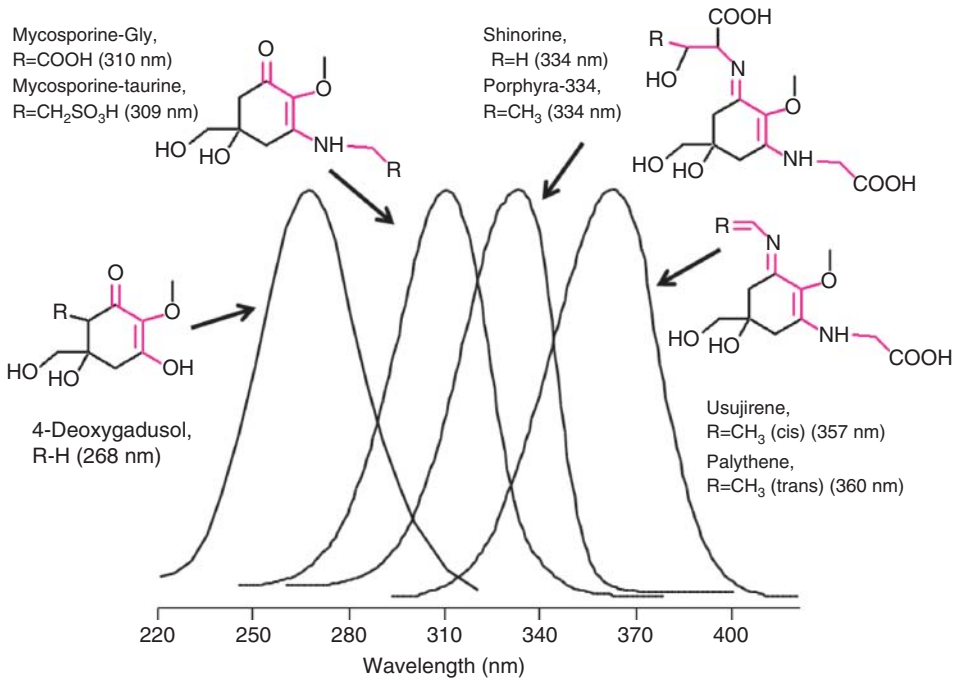


Plate 8.5 The mycosporines (MAAs); absorption spectrum and structure of representative compounds.

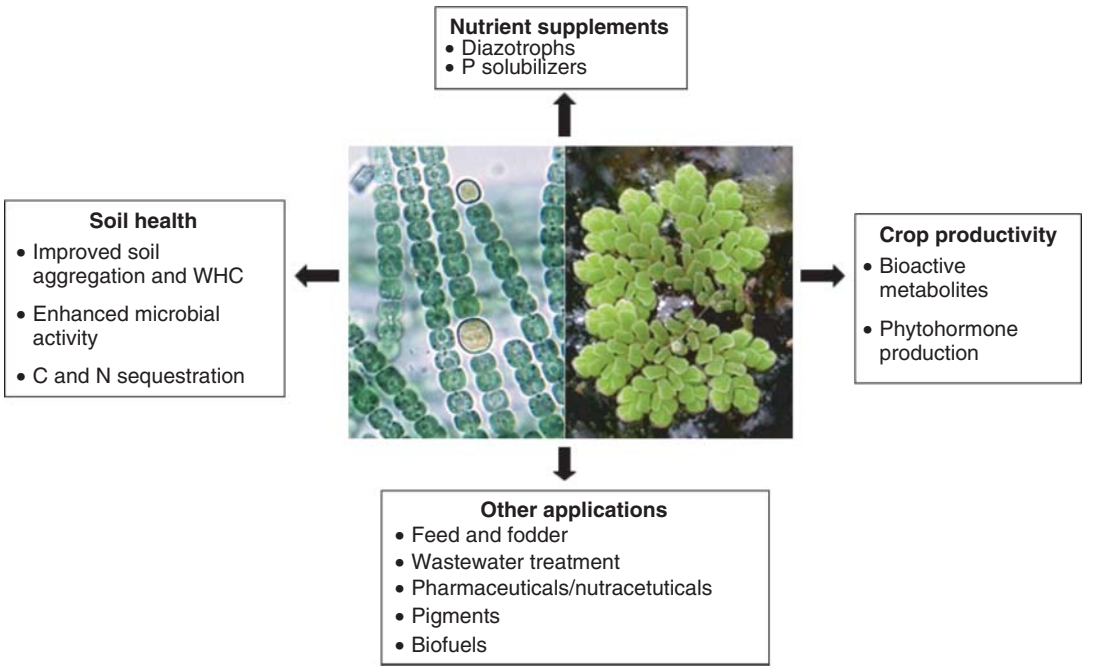


Plate 9.1 Overview of cyanobacteria and *Azolla* applications.

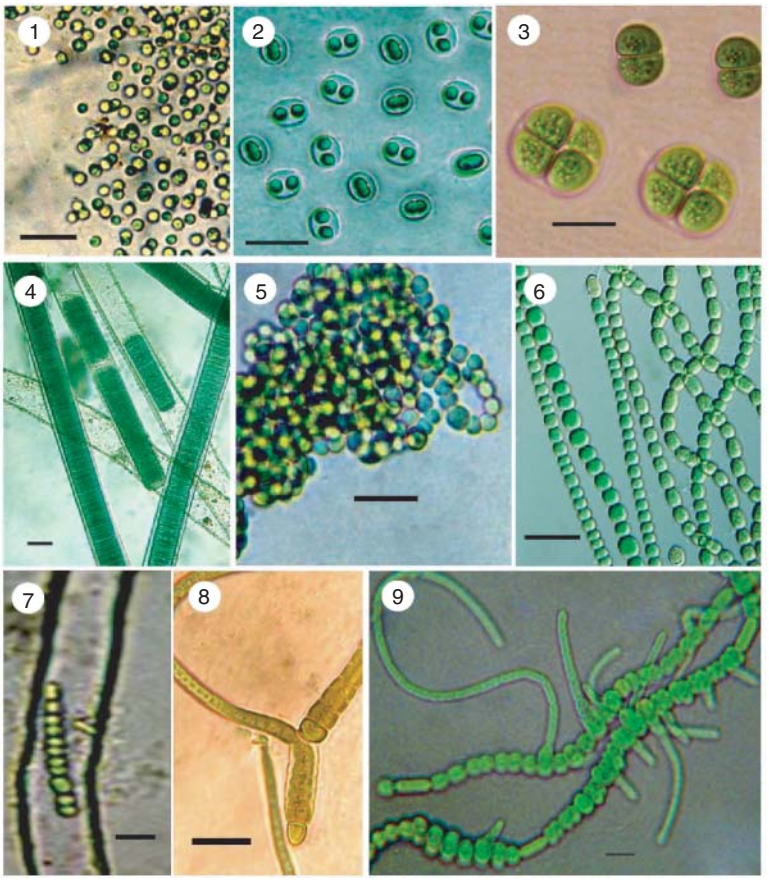


Plate 9.2 Microphotographs of cyanobacteria isolated from rice and wheat fields. (1) *Aphanocapsa* sp.; (2) *Gloeocapsa fusco-lutea*, (3) *Chroococcus turgidus* var. *maximus*, (4) *Lyngbya magnifica*, (5) *Nostoc commune*, (6) *Anabaena* sp., (7) filament of *Anabaena* sp. inside the wheat roots, (8) *Calothix parietina*, (9) *Westiellopsis prolifica* (Bar indicates 20 μm in photographs 1–3, 6–9; 10 μm in 5, and 50 μm in 4).

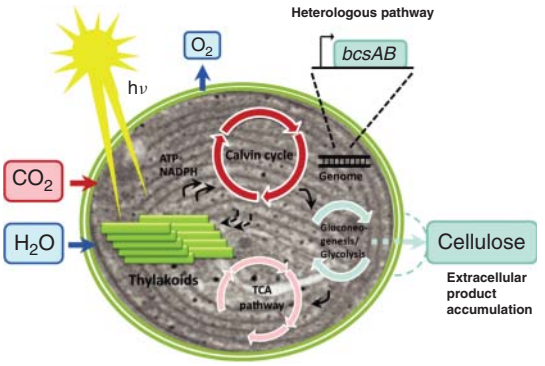


Plate 11.1 The cellulose synthesizing biosolar cell.

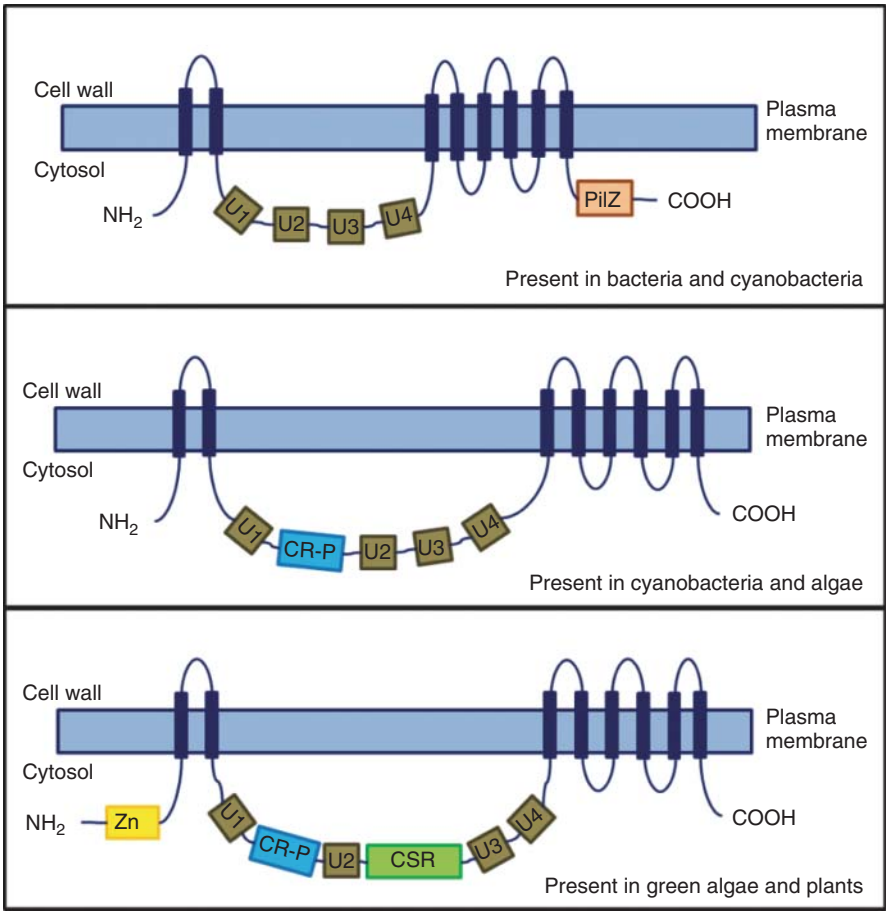


Plate 11.3 Membrane topology of cellulose synthase proteins. Blue bars indicate trans-membrane segments. PilZ, PilZ domain; Zn, RING-type zinc finger; U1–U4, catalytic domain; CR-P, plant specific region; CSR, class specific region. (Adapted from Taylor (2008). Reproduced with permission of John Wiley & Sons.)

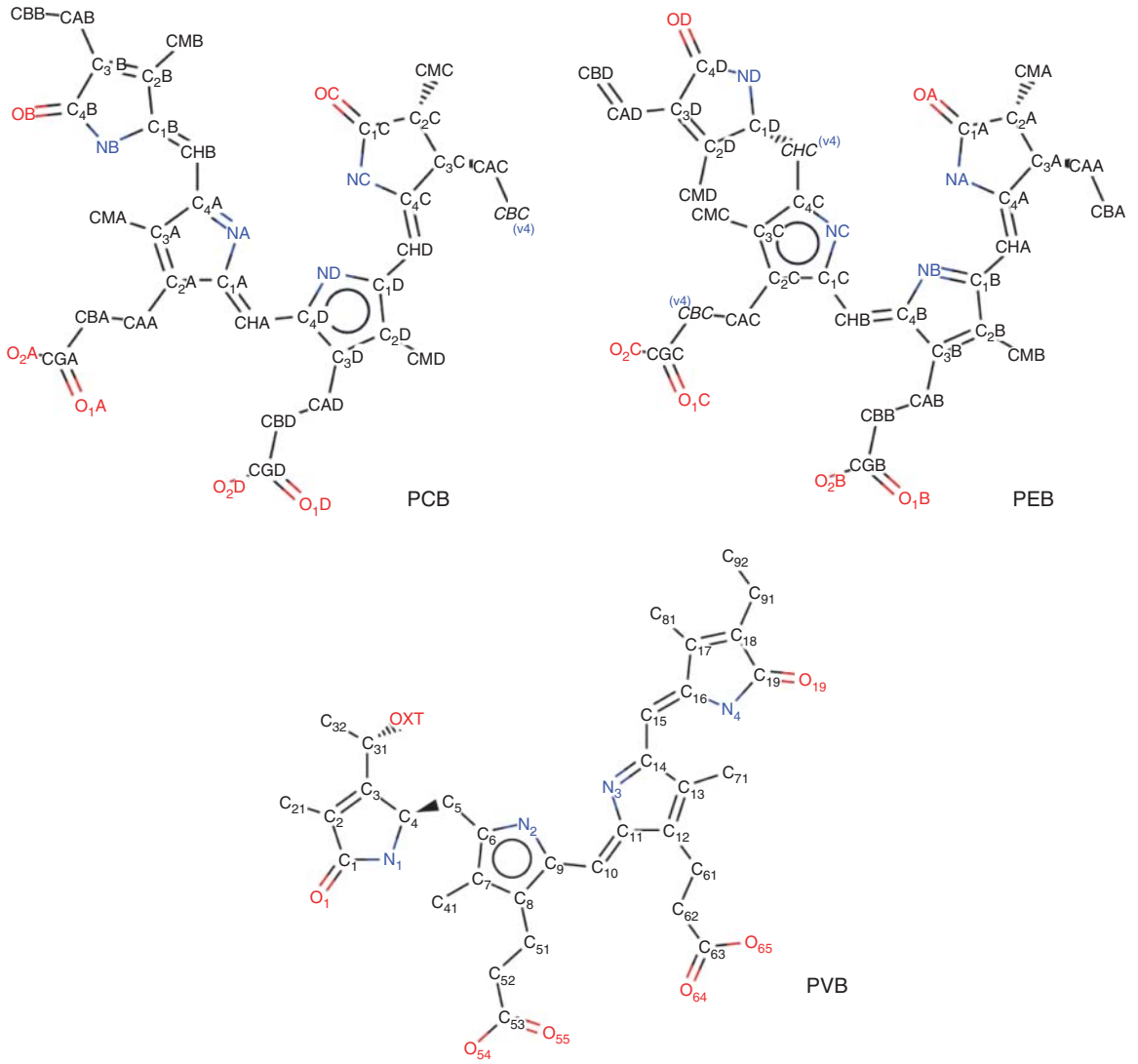
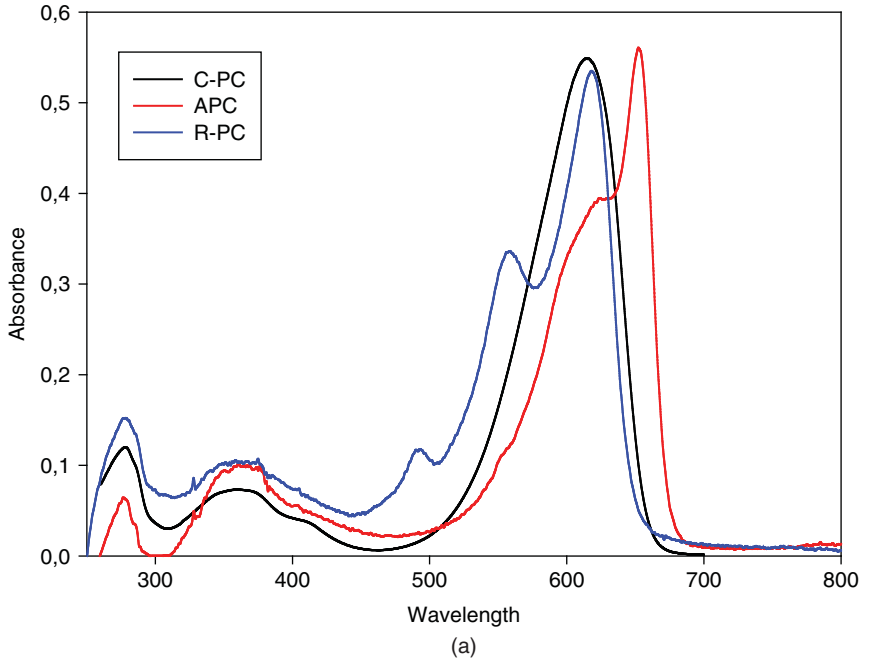


Plate 13.1 Structures of the different bilins (PCB, PEB, PVB) present in phycocyanins.



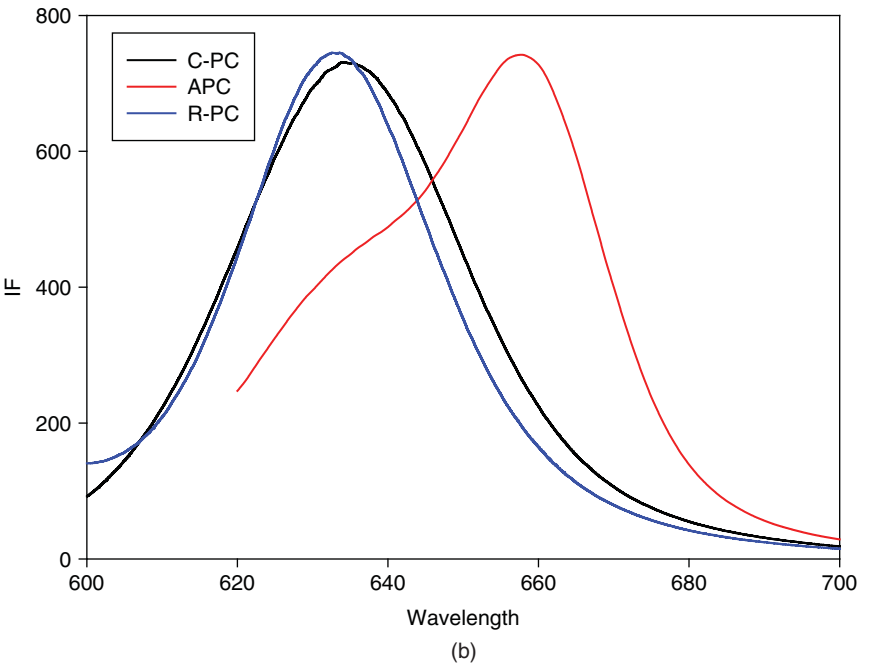


Plate 13.3 Spectroscopic characterization of C-PC (black line), APC (red line), and R-PC (blue line) in 5 mM sodium phosphate buffer, pH 7.0. (a) Absorbance spectra; (b), fluorescence emission spectra; (c) excitation fluorescence anisotropy spectra. The anisotropy values are the arithmetical average of five anisotropy measurements and the error bars are the standard deviations of these anisotropy determinations.

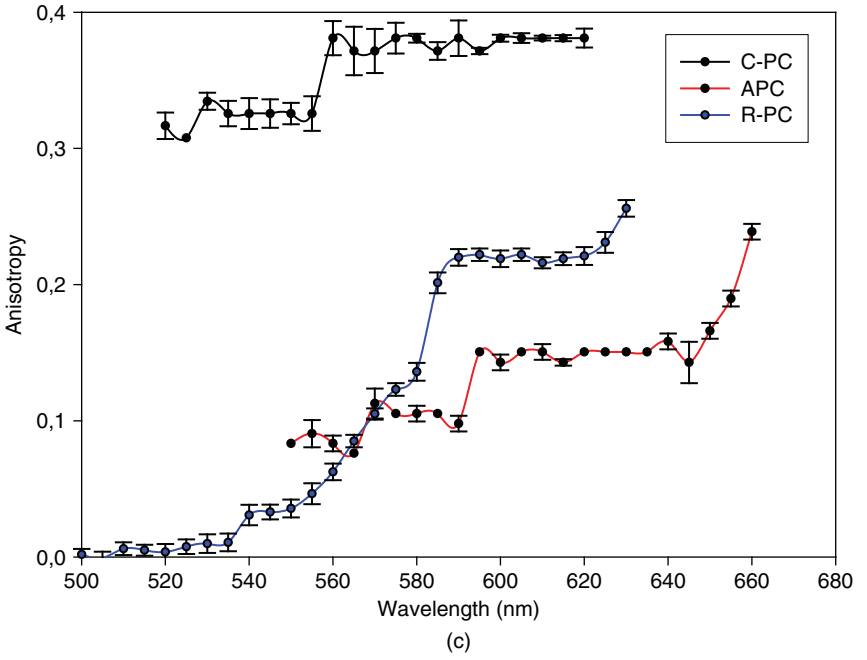


Plate 13.3 (*continued*)

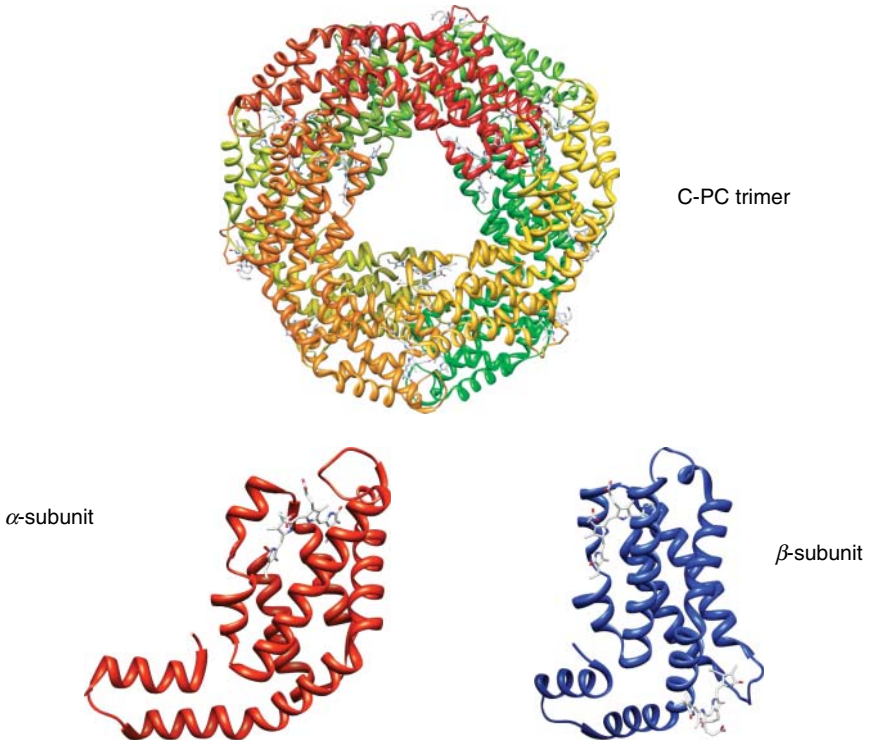


Plate 13.4 Locations and conformations of the nine phycocyanobilins in the crystal structure of the *Spirulina platensis* C-PC trimer and its subunits.

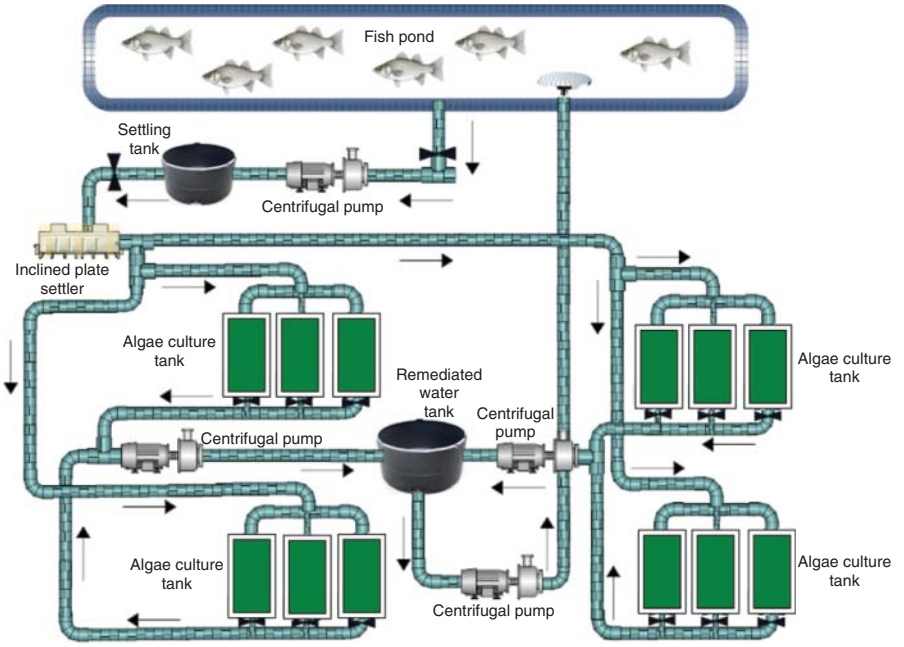


Plate 14.1 Diagrammatic representation of recirculatory aquaculture system.

Plate 14.2 View of culture tanks with *Aulosira fertilissima*.

Plate 14.3 View of photobioreactor with *Nostoc muscorum* Agardh.

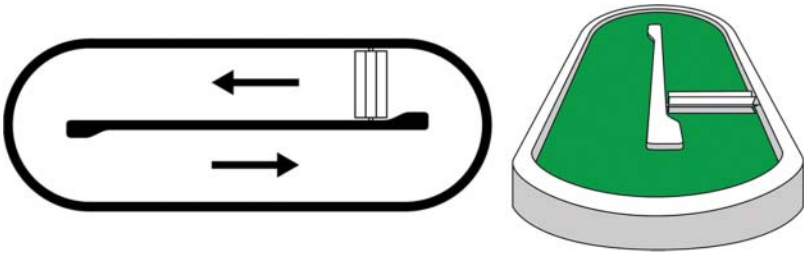


Plate 18.1 Schematic diagram of a raceway pond.

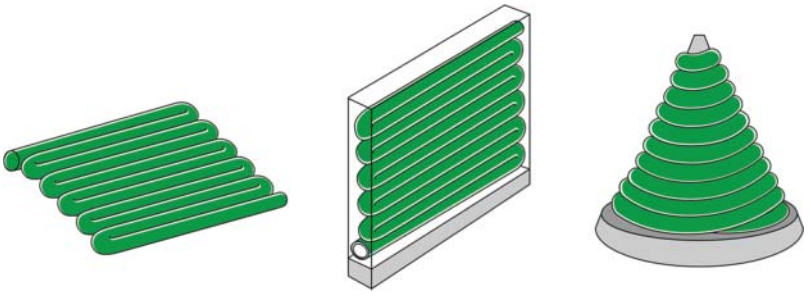


Plate 18.2 Tubular photobioreactors. The tubes can be placed parallel to each other lying flat above the ground (a), horizontal tubes can be arranged in parallel (b), or tubes made up of flexible plastic can be coiled around a supporting frame to form a helical-coil tubular photobioreactor (c).

(a) (b)

Plate 18.3 A column photobioreactor. Column photobioreactors can be suspended from a framework or supported within a mesh frame (a), or they can be rigid vertical columns (b).

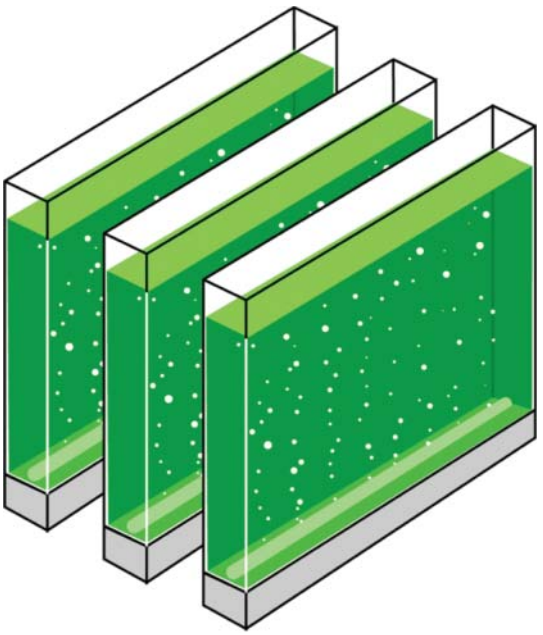


Plate 18.4 A flat panel photobioreactor.

Plate 18.5 A raceway pond in Miyako Island, Japan.

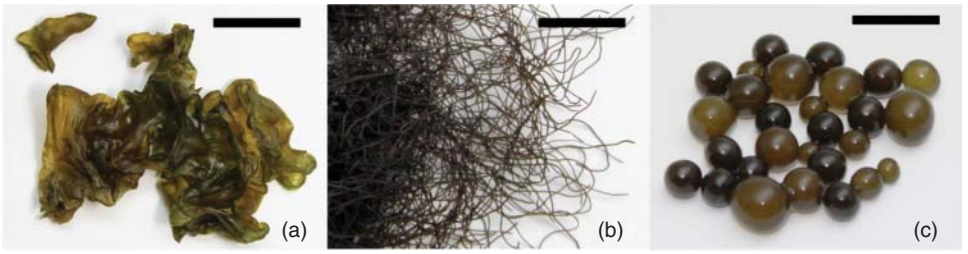


Plate 18.6 (a) *N. commune*; (b) *N. flagelliforme*; (c) *N. sphaericum* (scale bar = 1 cm).

Plate 18.7 A 200-l column photobioreactor.